

Elucidating the mechanism of glutamate transporters using magnetic resonance spectroscopy

Shona Hepworth

This thesis is submitted for the fulfilment of the degree of Doctor of
Philosophy

School of Chemistry
University of East Anglia
Norwich, UK

Submitted September 2023

This copy of the thesis has been supplied on condition that anyone who consults it is understood to recognise that its copyright rests with the author and that use of any information derived there-from must be in accordance with current UK Copyright Law. In addition, any quotation or extract must include full attribution.

Declaration

I certify that the work contained in this thesis submitted, by me, for the degree of Doctor of Philosophy, is my own original work, except where due reference is made to other authors or co-workers and has not previously been submitted, by me, for a degree at this or any other university.

Shona Hepworth,

September 2023

Acknowledgements

The last four years have included some of the best times of my life so far. I'd like to acknowledge some of the people who have contributed to my PhD journey.

My first thank you goes to my supervisor Dr Fraser MacMillan for the opportunity to complete a PhD in his laboratory, and to Dr Tharin Blumenschein for her support and guidance particularly over the last couple of years. A particular thank you and mention goes to Dr Justin Bradley for his scientific wisdom throughout the years and his hours of feedback and discussion of my writing.

I would also like to extend my thanks to the institutions and funders who financially supported this body of work and infrastructure: University of East Anglia (Department of Chemistry), the Norwich Research Park doctoral training partnership funded by BBSRC and to my iCASE partner Peak Proteins. Next, to my collaborators whom I have worked with to carry out this research: Dr Albert Guskov for providing guidance, plasmids, and the functional assays from his research group; Dr Chris Mulligan for providing plasmids and to Prof Eurico Cabrita for his discussion of the NMR experiments.

I have been at the University of East Anglia for the last 8 years and have met several people along the way. Specifically, Dr Jenny Smith– your friendship, support and guidance has been invaluable. Thanks to (almost Dr) Lizzie Gray for sharing both my difficulties and successes along the way. I want to thank my family for their love and ongoing support – I finally did it! This includes my two cats Lilly and Ralph for their unlimited love and affection. And lastly, the warmest thanks to my now husband Alex – my biggest supporter and the best partner I could ask for. I look forward to wherever the future takes me with you by my side.



Abstract

The homotrimeric aspartate membrane transporters from *Thermococcus kodakarensis* (Glt_{Tk}) and *Pyrococcus horikoshii* (Glt_{Ph}) are archaeal homologues of mammalian glutamate transporters belonging to the Solute Carrier 1 (SLC1) family which includes the mammalian excitatory amino acid transporters (EAATs). Dysfunction of these systems can lead to neurological disorders such as schizophrenia, Alzheimer's disease, and epilepsy. The EAATs regulate neurotransmitter levels in the synaptic cleft to prevent over-stimulation. Glt_{Ph} is often used as a model system for the EAATs and is well studied, however, Glt_{Tk} has been less well characterised especially with biophysical spectroscopic techniques. This thesis works to further elucidate the proposed alternating access mechanism of the EAATs using a range of magnetic resonance techniques.

Site-directed spin labelling using the cysteine specific spin probe MTSL has been used to study the functional dynamics of Glt_{Tk} during the transport cycle. Continuous wave (cw) EPR spectroscopy was used to investigate the changes in local environment of the spin probe, and double electron-electron resonance (DEER) spectroscopy to follow conformational changes during the transport cycle. Different membrane mimetics were studied to investigate the importance of 'biologically relevant' environments on both protein conformation and dynamics. These included detergent micelles, proteoliposomes, nanodiscs and styrene maleic acid lipid particles (SMALPs). This work provides evidence of independent function of the three protomers in trimeric Glt_{Tk} with evidence of potential cooperativity or steric effects when inhibitor bound. Saturation-Transfer Difference (STD) NMR, a ligand-based NMR technique, was used to determine apparent K_D values for L-aspartate and L-glutamate with Glt_{Tk}.

An orthogonal labelling approach was developed to further investigate the functional dynamics of Glt_{Ph}. Successful Cu²⁺-NTA labelling was assessed using cw-EPR and DEER spectroscopy with the future aim of using advanced EPR (iDEER) methods to extract novel distance distributions.

Access Condition and Agreement

Each deposit in UEA Digital Repository is protected by copyright and other intellectual property rights, and duplication or sale of all or part of any of the Data Collections is not permitted, except that material may be duplicated by you for your research use or for educational purposes in electronic or print form. You must obtain permission from the copyright holder, usually the author, for any other use. Exceptions only apply where a deposit may be explicitly provided under a stated licence, such as a Creative Commons licence or Open Government licence.

Electronic or print copies may not be offered, whether for sale or otherwise to anyone, unless explicitly stated under a Creative Commons or Open Government license. Unauthorised reproduction, editing or reformatting for resale purposes is explicitly prohibited (except where approved by the copyright holder themselves) and UEA reserves the right to take immediate 'take down' action on behalf of the copyright and/or rights holder if this Access condition of the UEA Digital Repository is breached. Any material in this database has been supplied on the understanding that it is copyright material and that no quotation from the material may be published without proper acknowledgement.

Table of Contents

1	Introduction	1
1.1	What are membrane transporter proteins?	1
1.2	Studying membrane transporter proteins	3
1.2.1	Membrane mimetics	3
1.2.2	Complementary methods for studying membrane transporter proteins	5
1.3	EPR spectroscopy of secondary active membrane transporters.....	8
	EPR spectroscopy of PutP	8
	EPR spectroscopy of LeuT	10
	EPR spectroscopy of vSGLT	12
	EPR spectroscopy of Mhp1	14
	EPR spectroscopy of BetP	15
1.4	Neurotransmission and the glutamate transporters	20
1.4.1	The SLC superfamily.....	20
1.4.2	Neurotransmission in the central nervous system.....	21
1.4.3	Excitatory amino acid transporters.....	23
1.5	Glt _{ph} and Glt _{tk} – archaeal homologues of the mammalian EAATs	28
1.5.1	General structure	29
1.5.2	Binding sites.....	31
1.5.3	Structural studies and biophysical characterisation of Glt _{tk} and Glt _{ph}	33
1.5.4	Previous studies of Glt _{ph} using EPR spectroscopy	44
1.5.5	The proposed transport mechanism	46
1.6	Aims and objectives	50
2	An introduction to Magnetic Resonance	51
2.1	Continuous wave EPR spectroscopy	53
2.1.1	EPR Spectrum acquisition.....	53
2.1.2	Site-directed spin labelling of proteins.....	54
2.1.3	Rotational correlation time.....	55
2.2	Fundamental theory.....	56
2.2.1	The spin Hamiltonian.....	56
2.2.2	The electron Zeeman interaction	57
2.2.3	Spectroscopic g-factor	59
2.2.4	The nuclear Zeeman interaction.....	62
2.2.5	The hyperfine interaction	64
2.2.6	Electron dipole-dipole interactions	65
2.2.7	Multifrequency EPR	66
2.3	Pulsed EPR spectroscopy	66
2.3.1	Reference frames, magnetisation, and pulses.....	67
2.3.2	Free induction decay and off-resonances.....	69
2.3.3	The primary two-pulse echo.....	70
2.3.4	Relaxation processes	71
2.3.5	The double electron-electron resonance (DEER) experiment.....	75
2.3.6	Interpreting the data	78
2.4	Fundamental theory of protein NMR spectroscopy	81
2.5	Saturation transfer difference (STD) NMR.....	83
3	Investigating the binding induced conformational changes of Glt_{tk} using continuous wave EPR spectroscopy	88

3.1	Rationale for the choice of labelling sites	88
3.2	<i>In silico</i> molecular modelling of Glt_{Tk}.....	92
3.3	Glt_{Tk} protein production, purification, and spin-labelling	96
3.4	Studying the conformational changes of Glt_{Tk} S281C using cw-EPR spectroscopy	100
3.4.1	Functional studies of Glt _{Tk} S281C in detergent micelles using cw-EPR	100
3.4.2	Stability of spin labelled Glt _{Tk} S281C in detergent micelles.....	102
3.4.3	Spin labelled Glt _{Tk} S281C studied in more biologically relevant environments	103
3.4.4	Studying immobilised spin-labelled Glt _{Tk} S281C using cw-EPR spectroscopy at X-band (9.4 GHz).....	106
3.4.5	Studying immobilised spin-labelled Glt _{Tk} S281C using cw-EPR spectroscopy at Q-band (34 GHz)	107
3.4.6	Spectral simulations of Glt _{Tk} S281C using EasySpin	107
3.5	Studying the conformational changes of Glt_{Tk} D298C using cw-EPR spectroscopy	121
3.5.1	Functional studies of Glt _{Tk} D298C in detergent micelles and proteoliposomes using cw-EPR	121
3.5.2	Spectral simulations of Glt _{Tk} D298C using EasySpin	123
3.6	Studying the conformational changes of Glt_{Tk} I312C using cw-EPR spectroscopy	130
3.6.1	Functional studies of Glt _{Tk} I312C in detergent micelles and proteoliposomes using cw-EPR	130
3.6.2	Spectral simulations of Glt _{Tk} I312C using EasySpin.....	131
3.7	Studying the conformational changes of Glt_{Tk} M314C using cw-EPR spectroscopy.....	137
3.7.1	Functional studies of Glt _{Tk} M314C in detergent micelles and proteoliposomes using cw-EPR	137
3.7.2	Spectral simulations of Glt _{Tk} M314C using EasySpin.....	139
3.8	Studying the conformational changes of Glt_{Tk} H334C using cw-EPR spectroscopy	144
3.8.1	Functional studies of Glt _{Tk} H334C in detergent micelles and proteoliposomes using cw-EPR	144
3.8.2	Spectral simulations of Glt _{Tk} H334C using EasySpin	145
3.9	Studying the conformational changes of Glt_{Tk} A367C using cw-EPR spectroscopy	150
3.9.1	Functional studies of Glt _{Tk} A367C in detergent micelles, proteoliposomes and SMALPs using cw-EPR	150
3.9.2	Spectral simulations of Glt _{Tk} A367C using EasySpin	152
3.10	Summary of results for Glt_{Tk} each binding state.....	159
3.11	Studying WT Glt_{Tk} as a control	162
4	<i>Studying the conformational changes of Glt_{Tk} during the transport cycle using pulsed EPR spectroscopy</i>	166
4.1	Initial pulsed EPR optimisation experiments.....	166
4.2	Measuring distance changes of Glt_{Tk} S281C using pulsed EPR spectroscopy ..	169
4.2.1	Functional studies of Glt _{Tk} S281C in detergent micelles.....	169
4.2.2	Functional studies of Glt _{Tk} S281C in more native environments.....	176
4.3	Measuring distance changes of Glt_{Tk} D298C using pulsed EPR spectroscopy .	179

4.4	Measuring distance changes of Glt _{7k} I312C using pulsed EPR spectroscopy ...	183
4.5	Measuring distance changes of Glt _{7k} M314C using pulsed EPR spectroscopy .	187
4.6	Measuring distance changes of Glt _{7k} H334C using pulsed EPR spectroscopy .	188
4.7	Measuring distance changes of Glt _{7k} A367C using pulsed EPR spectroscopy..	191
5	Utilising engineered double histidine motifs for copper (II) EPR studies of Glt_{ph}	195
5.1	Cu ²⁺ EPR theory	197
5.2	<i>In silico</i> molecular modelling of Glt _{ph}	198
5.3	Glt _{ph} sample preparation	202
5.4	EPR studies of MTSL labelled Glt _{ph}	203
5.4.1	Continuous wave EPR spectroscopy of Glt _{ph} labelled with MTSL.....	203
5.4.2	Pulsed EPR spectroscopy of MTSL labelled Glt _{ph} for distance determination	205
5.5	Spin labelling Glt _{ph} using Cu ²⁺ -NTA	208
5.5.1	His-cleavage	208
5.5.2	Using UV-Visible spectroscopy to check the formation of Cu ²⁺ -NTA.....	211
5.5.3	Spin labelling Glt _{ph} CM7 with Cu ²⁺ -NTA.....	212
5.6	Low temperature cw-EPR studies of Glt _{ph} spin labelled with Cu ²⁺ -NTA.....	212
5.6.1	Analysis of the formation of Cu ²⁺ -NTA.....	212
5.6.2	Cu ²⁺ -NTA labelled Glt _{ph} CM7	213
5.6.3	Orthogonally labelled (MTSL and Cu ²⁺ -NTA) Glt _{ph} CM7.....	214
5.7	Pulsed EPR spectroscopy of Cu ²⁺ -NTA labelled Glt _{ph} for distance determination	218
5.7.1	Confirming successful binding of Cu ²⁺ -NTA to dHis motif using pulsed EPR spectroscopy	218
5.7.2	Initial DEER studies using Cu ²⁺ -NTA as a label for Glt _{ph} CM7	219
6	Using STD NMR spectroscopy to determine ligand dissociation constants, K_D	224
6.1	WT Glt _{7k} STD NMR studies	225
6.1.1	WT Glt _{7k} STD-NMR direct titration with L-glutamate	225
6.1.2	WT Glt _{7k} STD-NMR competition titration with L-aspartate	230
6.1.3	WT Glt _{7k} STD-NMR direct titration with L-aspartate	234
6.2	Glt _{ph} STD NMR studies.....	235
6.2.1	WT Glt _{ph} STD-NMR titration with L-glutamate at varying sodium ion concentrations	235
6.2.2	STD-NMR titration with L-glutamate using Glt _{ph} C312S A71C with and without spin label	235
7	Discussion	237
7.1	Studying the conformational changes of Glt _{7k} using EPR spectroscopy.....	237
7.2	Investigating the binding interactions of Glt _{7k} with L-glutamate	246
7.3	Investigating the effect of Na ⁺ concentration on protein conformation.....	249
7.4	Orthogonal labelling strategies for Glt _{ph} to achieve narrower distance distributions	249
7.5	Summary and future work.....	252
8	Materials and methods.....	257

8.1	Glt_{TK}	257
8.1.1	Plasmids	257
8.1.2	Expression, purification, and site-directed spin labelling of Glt _{TK} in detergent.....	257
8.1.3	Functionality of spin labelled variants.....	259
8.1.4	Detergent-free environments	260
8.2	Glt_{PH}	264
8.2.1	Plasmids	264
8.2.2	Expression, purification, and site-directed spin labelling of Glt _{PH} in detergent	264
8.2.3	His-tag cleavage	265
8.3	Preparation and analysis of Copper (II) NTA	265
8.3.1	Formation of copper (II) NTA	265
8.3.2	UV-Visible spectroscopy of copper (II) NTA	265
8.3.3	Spin labelling Glt _{PH} with copper (II) NTA	266
8.4	EPR Spectroscopy	266
8.4.1	Data acquisition	266
8.4.2	Data analysis	269
8.5	NMR spectroscopy	271
8.5.1	Sample preparation	271
8.5.2	Data acquisition	271
9	Bibliography	273
Appendix		290
A1	K_D values of Glt_{TK} and Glt_{PH} for L-glutamate and L-aspartate	290
A2	Glt_{TK} uptake assay and ITC results (by Emanuela Colucci and Zaid Anshari)	291
A3	Sequence alignment of GltTK, GltPH and EAAT3	296
A4	Glt_{TK} MMM results visualised as chimera images	297
A5	EasySpin spectral simulations of immobilised Glt_{TK} S281C	299
A5.1	EasySpin simulation results for Glt _{TK} S281C in detergent micelles at 50K	299
A5.2	EasySpin simulation results for cw Glt _{TK} S281C in liposomes at 50K	300
A5.3	EasySpin simulation results for Glt _{TK} S281C in SMALPs at 50K	301
A5.4	EasySpin simulation results for Glt _{TK} S281C in nanodiscs at 50K	302
A6	EasySpin spectral simulations of immobilised Glt_{TK} D298C	303
A6.1	EasySpin simulation results for Glt _{TK} D298C in detergent micelles at 50K	303
A6.2	EasySpin simulation results for Glt _{TK} D298C in liposomes at 50K	304
A7	EasySpin spectral simulations of immobilised Glt_{TK} I312C	305
A7.1	EasySpin simulation results for Glt _{TK} I312C in detergent micelles at 50K	305
A7.2	EasySpin simulation results for Glt _{TK} I312C in liposomes at 50K	306
A8	Functional studies of Glt_{TK} M314C in detergent micelles and proteoliposomes using cw-EPR 307	
A9	EasySpin spectral simulations of immobilised Glt_{TK} M314C	308
A9.1	EasySpin simulation results for Glt _{TK} M314C in detergent micelles at 50K	308
A9.2	EasySpin simulation results for Glt _{TK} M314C in liposomes at 50K	309
A10	EasySpin spectral simulations of immobilised Glt_{TK} H334C	310
A10.1	EasySpin simulation results for Glt _{TK} H334C in detergent micelles at 50K	310
A10.2	EasySpin simulation results for Glt _{TK} H334C in liposomes at 50K	311
A11	EasySpin spectral simulations of immobilised Glt_{TK} A367C	312
A11.1	EasySpin simulation results for Glt _{TK} A367C in detergent micelles at 50K.....	312

A11.2 EasySpin simulation results for Glt _{TK} A367C in liposomes at 50K	313
A11.3 EasySpin simulation results for Glt _{TK} A367C in SMALPs at 50K	314
A12 Addition of extra L-aspartate to Glt_{TK} S281C in SMALPs	315
A13 The effect of adding d₈-glycerol on the T₂ spectrum of Glt_{TK} M314C	315
A14 Glt_{TK} M314C pulse EPR spectroscopy for distance determination in detergent micelles and liposomes	316
A15 Inversion recovery filtered DEER (iDEER)	317
A16 <i>In silico</i> modelling of Glt_{ph} using MMM.....	318
A17 <i>In silico</i> attachment of Cu²⁺-NTA to an engineered dHis motif on Glt_{ph} using Chimera and MMM	324
A18 Continuous wave EPR spectroscopy of Glt_{ph}.....	325
A19 Glt_{ph} 4-pulse DEER results for CM3, CM8, CM10 and CM11.....	329
A19.1 Glt _{ph} CM3 DEER results.....	329
A19.2 Glt _{ph} CM8 DEER results.....	330
A19.3 Glt _{ph} CM10 DEER results.....	332
A19.4 Glt _{ph} CM11 DEER results.....	333
A20 Continuous wave EPR spectroscopy of Glt_{ph}: His-tag cleavage control check	335
A21 A typical LT X-band spectrum of Cu.....	336
A22 The T₂ spectrum of Glt_{ph} CM7 spin-labelled with MTSL, Cu²⁺-NTA and orthogonally labelled.....	337

List of figures

Figure 1.1 Schematic representation of the different types of membrane transporter proteins.....	2
Figure 1.2 Membrane mimetics for structural and functional studies shown in increasing order of membrane complexity.....	4
Figure 1.3 The structure of styrene maleic acid (SMA) polymer	5
Figure 1.4 The proposed transport model of LeuT	12
Figure 1.5 The proposed sugar transport mechanism of vSGLT.....	13
Figure 1.6 Investigation of conformational changes of Mhp1 on addition of sodium and substrate	14
Figure 1.7 DEER studies of BetP using single and double cysteine variants.....	16
Figure 1.8 The glutamatergic synapse and the role of the EAATs in glutamate regulation in the synaptic cleft.	22
Figure 1.9 EAATs 1-3 in an inward facing conformation.	25
Figure 1.10 The three models of an alternating access mechanism for substrate transport across a membrane.....	26
Figure 1.11 Comparison of the transport stoichiometry in mammalian EAATs with archaeal glutamate transporters, Glt _{TK} and Glt _{Ph}	29
Figure 1.12 The mammalian EAAT and archaeal glutamate transporters, Glt _{TK} and Glt _{Ph} , membrane topology	31

Figure 1.13 Cartoon representation of the sodium ion and substrate binding sites of Glt _{TK} .	33
Figure 1.14 Structures of L-glutamate, L-aspartate and DL-threo- β -Benzyloxyaspartic (DL-TBOA).	34
Figure 1.15 Superposition of homotrimeric Glt _{TK} in an inward and outward facing conformation.	35
Figure 1.16 Glt _{TK} cryo-EM structures in different conformational states highlighting the asymmetry of the homotrimeric protein.	39
Figure 1.17 A proposed alternating access mechanism for Glt _{Ph} highlighting the gating mechanism on both sides of the membrane.	48
Figure 1.18 The coupled binding of sodium and aspartate by Glt _{Ph} during the alternating access mechanism via two proposed pathways.	49
Figure 2.1 Absorption of electromagnetic radiation leads to a transition between two discrete energy states.	51
Figure 2.2 The chemical structure of the spin label, MTSL, which reacts specifically with cysteine residues on protein molecules to form a disulfide bond.	55
Figure 2.3 The effect of rotational correlation times on the spectral line shape of an EPR spectrum.	56
Figure 2.4 The electron Zeeman interaction shown for an $S = \frac{1}{2}$ system.	58
Figure 2.5 The electron spin in a magnetic field with the population of the upper and lower spin levels displayed.	59
Figure 2.6 Three types of symmetry descriptions for an $S = \frac{1}{2}$ system: isotropic, axial and rhombic.	62
Figure 2.7 Energy level diagram of an electron ($S = \frac{1}{2}$) interacting with a proton ($I = \frac{1}{2}$).	63
Figure 2.8 A (frozen) nitroxide spectrum at two different microwave frequencies: X-band (9.75 GHz) and W-band (95 GHz).	66
Figure 2.9 The movement of an electron placed in a magnetic field and the resultant stationary magnetisation.	68
Figure 2.10 Bulk magnetisation vector (a) is tipped to the -y axis by a $\pi/2$ pulse.	69
Figure 2.11 The primary two-pulse echo.	71
Figure 2.12 The effect of pulses on the population states of the two energy states for an $S = \frac{1}{2}$ system (α and β).	72
Figure 2.13 The two relaxation mechanisms (a) Spin lattice longitudinal relaxation (T_1) and (b) spin-spin transverse relaxation (T_2).	73
Figure 2.14 The pulse sequence for an inversion recovery experiment for T_1 relaxation time determination.	73
Figure 2.15 Homogeneous and inhomogeneous broadening.	74
Figure 2.16 Schematic of the 3-pulse DEER sequence and the 4-pulse DEER sequence.	76
Figure 2.17 A field sweep echo (FSE) spectrum which is used to determine the optimal positions for pump and observe in the DEER experiment.	78
Figure 2.18 Processing DEER data using DeerAnalysis software within MATLAB.	79
Figure 2.19 Analysis of DEER data using DeerNet.	80
Figure 2.20 An energy diagram for a two-spin system.	84
Figure 2.21 The STD NMR experiment.	85
Figure 2.22 Saturation transfer difference (NMR) schematic.	86

Figure 2.23 Ligand epitope mapping..	87
Figure 3.1 Glt _{Tk} membrane topology..	89
Figure 3.2 MMM results for Glt _{Tk} variants.....	90
Figure 3.3 SDS PAGE of Glt _{Tk} A367C purified using cobalt TALON Metal Affinity Resin and Ni Sepharose 6 Fast Flow resin	96
Figure 3.4 X-band RT cw-EPR spectra of apo Glt _{Tk} S281C following SDSL during purification and after purification.....	97
Figure 3.5 X-band RT cw-EPR spectra of TEMPO	98
Figure 3.6 X-band RT cw-EPR spectra of all six Glt _{Tk} variants in an apo state	99
Figure 3.7 X-band RT cw-EPR spectra of Glt _{Tk} S281C in detergent micelles utilising substrate and co-transported Na ⁺ to trap Glt _{Tk} in proposed intermediates of the transport cycle..	101
Figure 3.8 X-band RT cw-EPR spectra of Glt _{Tk} S281C in detergent micelles using Na ⁺ concentrations of 50 mM and 300 mM.	102
Figure 3.9 The evolution of the free-MTSL signal in apo Glt _{Tk} S281C over time.	103
Figure 3.10 X-band RT cw-EPR spectra of apo Glt _{Tk} S281C in different membrane mimetics	105
Figure 3.11 X-band LT cw-EPR spectra (at 50 K) of apo Glt _{Tk} S281C in different membrane mimetics	106
Figure 3.12 LT Q-band cw-EPR spectra for Glt _{Tk} S281C in detergent micelles and their respective simulations using EasySpin	109
Figure 3.13 LT Q-band and LT X-band cw-EPR spectra for apo Glt _{Tk} S281C in detergent micelles and their respective simulations using a multifrequency approach in EasySpin	110
Figure 3.14 RT X-band cw-EPR spectra for Glt _{Tk} S281C in detergent micelles and their respective simulations using EasySpin	113
Figure 3.15 RT X-band cw-EPR spectra for Glt _{Tk} S281C in proteoliposomes and their respective simulations using EasySpin.....	115
Figure 3.16 RT X-band cw-EPR spectra for Glt _{Tk} S281C in SMALPs and their respective simulations using EasySpin	116
Figure 3.17 RT X-band cw-EPR spectra for Glt _{Tk} S281C in nanodiscs and their respective simulations using EasySpin	116
Figure 3.18 X-band RT cw-EPR spectra of Glt _{Tk} D298C in detergent micelles and proteoliposomes utilising substrate and co-transported Na ⁺ to trap Glt _{Tk} in proposed intermediates of the transport cycle	122
Figure 3.19 RT X-band cw-EPR spectra for Glt _{Tk} D298C in detergent micelles and their respective simulations using EasySpin	124
Figure 3.20 RT X-band cw-EPR spectra for Glt _{Tk} D298C in proteoliposomes and their respective simulations using EasySpin	127
Figure 3.21 X-band RT cw-EPR spectra of Glt _{Tk} I312C in detergent micelles and proteoliposomes utilising substrate and co-transported Na ⁺ to trap Glt _{Tk} in proposed intermediates of the transport cycle	131
Figure 3.22 RT X-band cw-EPR spectra for Glt _{Tk} I312C in detergent micelles and their respective simulations using EasySpin	132
Figure 3.23 RT X-band cw-EPR spectra for Glt _{Tk} I312C in proteoliposomes and their respective simulations using EasySpin.....	136

Figure 3.24 X-band RT cw-EPR spectra of Glt _{TK} M314C in detergent micelles utilising substrate and co-transported Na ⁺ to trap Glt _{TK} in proposed intermediates of the transport cycle	139
Figure 3.25 RT X-band cw-EPR spectra for Glt _{TK} M314C in detergent micelles and their respective simulations using EasySpin	140
Figure 3.26 RT X-band cw-EPR spectra for Glt _{TK} M314C in proteoliposomes and their respective simulations using EasySpin	142
Figure 3.27 X-band RT cw-EPR spectra of Glt _{TK} H334C in detergent micelles and proteoliposomes utilising substrate and co-transported Na ⁺ to trap Glt _{TK} in proposed intermediates of the transport cycle	145
Figure 3.28 RT X-band cw-EPR spectra for Glt _{TK} H334C in detergent micelles and their respective fits using EasySpin	146
Figure 3.29 RT X-band cw-EPR spectra for Glt _{TK} H334C in proteoliposomes and their respective fits using EasySpin	148
Figure 3.30 X-band RT cw-EPR spectra of Glt _{TK} A367C in detergent micelles utilising substrate and co-transported Na ⁺ to trap Glt _{TK} in proposed intermediates of the transport cycle	151
Figure 3.31 X-band RT cw-EPR spectra of Glt _{TK} A367C in detergent micelles, proteoliposomes and SMALPs utilising substrate and co-transported Na ⁺ to trap Glt _{TK} in proposed intermediates of the transport cycle.....	152
Figure 3.32 RT X-band cw-EPR spectra for Glt _{TK} A367C in detergent micelles and their respective simulations using EasySpin	153
Figure 3.33 RT X-band cw-EPR spectra for Glt _{TK} A367C in proteoliposomes and their respective simulations using EasySpin	158
Figure 3.34 RT X-band cw-EPR spectra for Glt _{TK} A367C in SMALP and their respective simulations using EasySpin	158
Figure 3.35 X-band RT cw-EPR spectra of apo WT Glt _{TK} following SDSL after purification and after being subjected to extra wash steps	162
Figure 3.36 RT X-band cw-EPR spectra for WT Glt _{TK} in detergent micelles and the simulation from EasySpin.....	163
Figure 3.37 The time decay trace of WT Glt _{TK} in a 4-pulse DEER experiment for distance determination.....	164
Figure 4.1 Optimisation of the pump and observe positions for DEER experiments of apo Glt _{TK} S281C..	167
Figure 4.2 Optimisation of the frequency offset for the pump and observe positions for DEER experiments of Glt _{TK} S281C apo.....	168
Figure 4.3 The effect of adding different viscous solutions (30% v/v) on the T ₂ experiment of apo Glt _{TK} D298C.....	169
Figure 4.4 X-band field swept echo (FSE) spectra measured for Glt _{TK} S281C in detergent micelles (50 K).	170
Figure 4.5 MMM results for Glt _{TK} S281C in an outward open (left, PDB 4KY0) and 2 inward open protomers with 1 intermediate outward occluded protomer (right, PDB 6XWR) with MTS rotamers shown as red clusters..	171
Figure 4.6 4-pulse DEER experiments of Glt _{TK} S281C in detergent micelles utilising substrate and co-transported Na ⁺ to trap Glt _{TK} in proposed intermediates of the transport cycle.	171

Figure 4.7 4-pulse DEER experiments of Glt _{Tk} S281C in detergent micelles at 50 mM Na ⁺ compared to 300 mM Na ⁺ .	174
Figure 4.8 4-pulse DEER experiments of Glt _{Tk} S281C in detergent micelles with 50 mM Na ⁺ and additions of 1 mM L-aspartate, 1 mM L-glutamate and 1 mM L-glutamate followed by 1 mM L-aspartate.	175
Figure 4.9 4-pulse DEER experiments of Glt _{Tk} S281C in proteoliposomes, SMALPs and nanodiscs utilising substrate and co-transported Na ⁺ to trap Glt _{Tk} in proposed intermediates of the transport cycle.	178
Figure 4.10 4-pulse DEER experiments of Glt _{Tk} D298C in detergent micelles utilising substrate and co-transported Na ⁺ to trap Glt _{Tk} in proposed intermediates of the transport cycle	180
Figure 4.11 MMM results for Glt _{Tk} D298C in an outward open (left, PDB 4KY0) and 2 inward open protomers with 1 intermediate outward occluded protomer (right, PDB 6XWR) with MTSL rotamers shown as red clusters..	181
Figure 4.12 4-pulse DEER experiments of Glt _{Tk} D298C in proteoliposomes utilising substrate and co-transported Na ⁺ to trap Glt _{Tk} in proposed intermediates of the transport cycle	182
Figure 4.13 The effects of glycerol on the T ₂ experiment of Glt _{Tk} I312C in an apo state.....	184
Figure 4.14 4-pulse DEER experiments of Glt _{Tk} I312C in detergent micelles and proteoliposomes utilising substrate and co-transported Na ⁺ to trap Glt _{Tk} in proposed intermediates of the transport cycle.....	185
Figure 4.15 MMM results for Glt _{Tk} I312C in an outward open (left, PDB 4KY0) and 2 inward open protomers with 1 intermediate outward occluded protomer (right, PDB 6XWR) with MTSL rotamers shown as red clusters..	186
Figure 4.16 MMM results for Glt _{Tk} M314C in an outward open (left, PDB 4KY0) and 2 inward open protomers with 1 intermediate outward occluded protomer (right, PDB 6XWR) with MTSL rotamers shown as red clusters..	187
Figure 4.17 MMM results for Glt _{Tk} H334C in an outward open (left, PDB 4KY0) and 2 inward open protomers with 1 intermediate outward occluded protomer (right, PDB 6XWR) with MTSL rotamers shown as red clusters..	189
Figure 4.18 4-pulse DEER experiments of Glt _{Tk} H334C in detergent micelles and proteoliposomes utilising substrate and co-transported Na ⁺ to trap Glt _{Tk} in proposed intermediates of the transport cycle.....	190
Figure 4.19 MMM results for Glt _{Tk} A356C in an outward open (left, PDB 4KY0) and 2 inward open protomers with 1 intermediate outward occluded protomer (right, PDB 6XWR) with MTSL rotamers shown as red clusters..	191
Figure 4.20 4-pulse DEER experiments of Glt _{Tk} A367C in detergent micelles, proteoliposomes and SMALPs utilising substrate and co-transported Na ⁺ to trap Glt _{Tk} in proposed intermediates of the transport cycle.....	193
Figure 5.1 Spin-labelling protein using MTSL and Cu ²⁺ -NTA.....	195
Figure 5.2 Binding of Cu ²⁺ to a dHis motif on alpha helix and beta sheet.....	196
Figure 5.3 The two coordination modes of Cu ²⁺ -NTA.....	197
Figure 5.4 Glt _{ph} membrane topology and structure.	199
Figure 5.5 X-band RT cw-EPR spectra of Glt _{ph} CM7 in detergent micelles utilising substrate and co-transported Na ⁺ to trap Glt _{ph} in proposed intermediates of the transport cycle.	204

Figure 5.6 4-pulse DEER experiments of MTSL-labelled Glt _{Ph} CM7 in detergent micelles utilising substrate and co-transported Na ⁺ to trap Glt _{Ph} in proposed intermediates of the transport cycle.	206
Figure 5.7 SDS PAGE of Glt _{Ph} CM7 to check the poly-histidine tag cleavage using thrombin.	208
Figure 5.8 X-band RT cw-EPR spectra of apo Glt _{Ph} CM3.	209
Figure 5.9 X-band LT cw-EPR spectra of Glt _{Ph} CM7 (at 50 K) with and without the poly-histidine tag attached.	210
Figure 5.10 Low-temperature cw-EPR spectrum of his-cleaved, MTSL-labelled Glt _{Ph} CM3 to show no advantageous metal binding.	211
Figure 5.11 UV-Vis spectra of CuCl ₂ and Cu ²⁺ -NTA in buffer at 300 nm and 800 nm	211
Figure 5.12 X-band LT cw-EPR spectra of CuCl ₂ and Cu ²⁺ -NTA in buffer at 10 K....	213
Figure 5.13 Investigating spin-labelling Glt _{Ph} CM7 dHis motif with Cu ²⁺ -NTA using X-band LT cw-EPR spectroscopy at 10 K.....	214
Figure 5.14 EasySpin simulations of LT cw-EPR spectra of Glt _{Ph} CM7 with MTSL and/or Cu ²⁺ -NTA at 10 K (or 50 K for MTSL-only).	216
Figure 5.15 The 3-pulse ESSEM sequence.	218
Figure 5.16 3-pulse ESSEM experiment of Cu ²⁺ -NTA in buffer and Cu ²⁺ -NTA labelled Glt _{Ph} CM7	219
Figure 5.17 Field swept echo experiment of Glt _{Ph} CM7 spin labelled with MTSL, Cu ²⁺ -NTA and orthogonally labelled with both.....	220
Figure 5.18 4-pulse DEER experiments of Cu ²⁺ -NTA labelled Glt _{Ph} CM7 in detergent micelles utilising substrate and co-transported Na ⁺ to trap Glt _{Ph} in proposed intermediates of the transport cycle..	221
Figure 5.19 4-pulse DEER experiments of apo Glt _{Ph} CM7 labelled with MTSL, Cu ²⁺ -NTA and orthogonally labelled with both.....	222
Figure 6.1 ¹ H NMR spectra recorded as control experiments with 1D ¹ H reference spectra (i.e. on-resonance) and STD spectra	227
Figure 6.2 ¹ H NMR spectra of detergent solubilised WT Glt _{Tk} (18 μM) in buffer containing 100 mM NaCl following incubation with 5 mM L-glutamate.....	228
Figure 6.3 A plot of the STD-AF against L-glutamate concentration for WT Glt _{Tk} (18 μM) with 100 mM NaCl.....	230
Figure 6.4 ¹ H NMR spectra of detergent solubilised WT Glt _{Tk} (25 μM) in buffer containing 100 mM NaCl following incubation with 3 mM L-glutamate (L-glu) and 3 mM L-aspartate (L-asp).....	231
Figure 6.5 A plot of the % bound (normalised STD effect) against L-aspartate concentration for the competition titration of WT Glt _{Tk} (16 μM) in the presence of 5 mM L-glutamate and 100 mM NaCl.....	232
Figure 6.6 A plot of the STD-AF against L-aspartate concentration for WT Glt _{Tk} (25 μM) with 100 mM NaCl.....	234
Figure 6.7 A plot of the STD-AF against L-glutamate concentration for WT Glt _{Ph} (20 μM) at 100 mM NaCl.....	235
Figure 6.8 A plot of the STD-AF against L-glutamate concentration for WT Glt _{Ph} (stars, 22 μM), Glt _{Ph} C321A A71C (circles, 23 μM) and Glt _{Ph} C321A A71C + MTSL (diamonds, 18 μM) with 100 mM NaCl.....	236

Figure 7.1 The proposed alternating access mechanism for the glutamate transporters and their identification using EPR spectroscopy.	254
Figure A1 Sodium ion dependency of Glt _{Tk} variants compared to WT using uptake assays.	292
Figure A2 Time course experiments of Glt _{Tk} variants compared to WT using uptake assays.	292
Figure A3 Comparison of Glt _{Tk} S281C with and without spin label (MTSL) using uptake assays.	293
Figure A4 Isothermal titration calorimetry (ITC) results for Glt _{Tk} S281C with and without spin-label.	294
Figure A5 Isothermal titration calorimetry (ITC) results for Glt _{Tk} D298C and H334C.	295
Figure A6 Sequence alignment of the aspartate transporters Glt _{Tk} from <i>Thermococcus kodakarensis</i> and Glt _{Ph} from <i>Pyrococcus horikoshii</i> ; and the glutamate transporter EAAT3 from <i>Rattus norvegicus</i>	296
Figure A7 MMM results for Glt _{Tk} variants	298
Figure A8 LT X-band cw-EPR spectra for Glt _{Tk} S281C in detergent micelles and their respective simulations using EasySpin for the proposed intermediates of the transport cycle	299
Figure A9 LT X-band cw-EPR spectra for Glt _{Tk} S281C in liposomes and their respective simulations using EasySpin for the proposed intermediates of the transport cycle	300
Figure A10 LT X-band cw-EPR spectra for Glt _{Tk} S281C in SMALPs and their respective simulations using EasySpin for the proposed intermediates of the transport cycle	301
Figure A11 LT X-band cw-EPR spectra for Glt _{Tk} S281C in nanodiscs and their respective simulations using EasySpin for the proposed intermediates of the transport cycle	302
Figure A12 LT X-band cw-EPR spectra for Glt _{Tk} D298C in detergent micelles and their respective simulations using EasySpin for the proposed intermediates of the transport cycle	303
Figure A13 LT X-band cw-EPR spectra for Glt _{Tk} D298C in liposomes and their respective simulations using EasySpin for the proposed intermediates of the transport cycle	304

Figure A14 LT X-band cw-EPR spectra for Glt _{Tk} I312C in detergent micelles and their respective simulations using EasySpin for the proposed intermediates of the transport cycle	305
Figure A15 LT X-band cw-EPR spectra for Glt _{Tk} I312C in liposomes and their respective simulations using EasySpin for the proposed intermediates of the transport cycle	306
Figure A16 X-band RT cw-EPR spectra of Glt _{Tk} M314C in detergent micelles and liposomes utilising substrate and co-transported Na ⁺ to trap Glt _{Tk} in proposed intermediates of the transport cycle	307
Figure A17 LT X-band cw-EPR spectra for Glt _{Tk} M314C in detergent micelles and their respective simulations using EasySpin for the proposed intermediates of the transport cycle	308
Figure A18 LT X-band cw-EPR spectra for Glt _{Tk} M314C in liposomes and their respective simulations using EasySpin for the proposed intermediates of the transport cycle	309
Figure A19 LT X-band cw-EPR spectra for Glt _{Tk} H334C in detergent micelles and their respective simulations using EasySpin for the proposed intermediates of the transport cycle	310
Figure A20 LT X-band cw-EPR spectra for Glt _{Tk} H334C in liposomes and their respective simulations using EasySpin for the proposed intermediates of the transport cycle	311
Figure A21 LT X-band cw-EPR spectra for Glt _{Tk} A367C in detergent micelles and their respective simulations using EasySpin for the proposed intermediates of the transport cycle	312
Figure A22 LT X-band cw-EPR spectra for Glt _{Tk} A367C in liposomes and their respective simulations using EasySpin for the proposed intermediates of the transport cycle	313
Figure A23 LT X-band cw-EPR spectra for Glt _{Tk} A367C in SMALPs and their respective simulations using EasySpin for the proposed intermediates of the transport cycle	314
Figure A24 4-pulse DEER experiments (50 K) for substrate-bound Glt _{Tk} S281C in SMALPs with the addition of excess L-aspartate	315
Figure A25 The effects of glycerol on the T ₂ experiment of Glt _{Tk} M314C in an apo state.....	315
Figure A26 4-pulse DEER experiments (50 K) for Glt _{Tk} M314C in detergent micelles and liposomes utilising substrate and co-transported Na ⁺ to trap Glt _{Tk} in proposed intermediates of the transport cycle	316
Figure A27 Pulse sequence for the iDEER experiment	317

Figure A28 Glt _{Ph} membrane topology highlighting the locations of the cysteine residues and dHis motif for each variant.	319
Figure A29 X-band RT cw-EPR spectra of Glt _{Ph} CM3 in detergent micelles utilising substrate and co-transported Na ⁺ to trap Glt _{Ph} in proposed intermediates of the transport cycle	325
Figure A30 X-band RT cw-EPR spectra of Glt _{Ph} CM8 in detergent micelles utilising substrate and co-transported Na ⁺ to trap Glt _{Ph} in proposed intermediates of the transport cycle	326
Figure A31 X-band RT cw-EPR spectra of Glt _{Ph} CM10 in detergent micelles utilising substrate and co-transported Na ⁺ to trap Glt _{Ph} in proposed intermediates of the transport cycle.	327
Figure A32 X-band RT cw-EPR spectra of Glt _{Ph} CM11 in detergent micelles utilising substrate and co-transported Na ⁺ to trap Glt _{Ph} in proposed intermediates of the transport cycle	328
Figure A33 4-pulse DEER experiments of MTSL-labelled Glt _{Ph} CM3 in detergent micelles utilising substrate and co-transported Na ⁺ to trap Glt _{Ph} in proposed intermediates of the transport cycle	330
Figure A34 4-pulse DEER experiments of MTSL-labelled Glt _{Ph} CM8 in detergent micelles utilising substrate and co-transported Na ⁺ to trap Glt _{Ph} in proposed intermediates of the transport cycle	331
Figure A35 4-pulse DEER experiments of MTSL-labelled Glt _{Ph} CM10 in detergent micelles utilising substrate and co-transported Na ⁺ to trap Glt _{Ph} in proposed intermediates of the transport cycle	332
Figure A36 4-pulse DEER experiments of MTSL-labelled Glt _{Ph} CM11 in detergent micelles utilising substrate and co-transported Na ⁺ to trap Glt _{Ph} in proposed intermediates of the transport cycle.	334
Figure A37 X-band RT cw-EPR spectra of all Glt _{Ph} variants in detergent micelles in an apo state and following poly-histidine tag cleavage	335
Figure A38 EPR spectrum of Cu ²⁺ at LT X-band	336
Figure A39 T ₂ experiment of Glt _{Ph} CM7 spin labelled with MTSL, Cu ²⁺ -NTA and orthogonally labelled with both.....	337

List of tables

Table 3.1 Results of MMM analysis using structural models of Glt _{Tk} spin labelled with MTSL.....	95
--	----

Table 3.2 Glt _{Tk} protein yield for each variant and the calculated spin labelling efficiency	98
Table 3.3 Best fit simulation values for LT Q-band cw-EPR spectra of Glt _{Tk} S281C in detergent micelles	109
Table 3.4 Best fit simulation values using the multifrequency simulation function for LT Q-band and LT X-band cw-EPR spectra of apo Glt _{Tk} S281C in detergent micelles	110
Table 3.5 Best fit simulation values for RT X-band cw-EPR spectra of Glt _{Tk} S281C in detergent micelles	112
Table 3.6 Best fit simulation values for RT X-band cw-EPR spectra of Glt _{Tk} S281C in proteoliposomes	117
Table 3.7 Best fit simulation values for RT X-band cw-EPR spectra of Glt _{Tk} S281C in SMALPs.....	118
Table 3.8 Best fit simulation values for RT X-band cw-EPR spectra of Glt _{Tk} S281C in nanodiscs.....	119
Table 3.9 Best fit simulation values for RT X-band cw-EPR spectra of Glt _{Tk} D298C in detergent micelles	126
Table 3.10 Best fit simulation values for RT X-band cw-EPR spectra of Glt _{Tk} D298C in proteoliposomes.	128
Table 3.11 Best fit simulation values for RT X-band cw-EPR spectra of Glt _{Tk} I312C in detergent micelles	133
Table 3.12 Best fit simulation values for RT X-band cw-EPR spectra of Glt _{Tk} I312C in proteoliposomes.	135
Table 3.13 Best fit simulation values for RT X-band cw-EPR spectra of Glt _{Tk} M314C in detergent micelles	141
Table 3.14 Best fit simulation values for RT X-band cw-EPR spectra of Glt _{Tk} M314C in proteoliposomes	143
Table 3.15 Best fit simulation values for RT X-band cw-EPR spectra of Glt _{Tk} H334C in detergent micelles	147
Table 3.16 Best fit simulation values for RT X-band cw-EPR spectra of Glt _{Tk} H334C in proteoliposomes.	149
Table 3.17 Best fit simulation values for RT X-band cw-EPR spectra of Glt _{Tk} A367C in detergent micelles	154
Table 3.18 Best fit simulation values for RT X-band cw-EPR spectra of Glt _{Tk} A367C in proteoliposomes	156
Table 3.19 Best fit simulation values for RT X-band cw-EPR spectra of Glt _{Tk} A367C in SMALPs.....	157

Table 3.20 A summary of EasySpin fitting results for apo Glt _{Tk} variants and the corresponding protein conformations	160
Table 3.21 A summary of EasySpin fitting results for Na ⁺ bound Glt _{Tk} variants and the corresponding protein conformations	160
Table 3.22 A summary of EasySpin fitting results for Na ⁺ -substrate bound Glt _{Tk} variants and the corresponding protein conformations	161
Table 3.23 A summary of EasySpin fitting results for Na ⁺ -inhibitor bound Glt _{Tk} variants and the corresponding protein conformations	161
Table 3.24 Best fit simulation values for RT X-band cw-EPR spectra of WT Glt _{Tk} detergent micelles	163
Table 4.1 A summary of the distance distributions calculated (using DeerNet) from the 4-pulse DEER traces of Glt _{Tk} S281C.....	176
Table 5.1 Results for MMM analysis of Glt _{Ph} CM7 (C321S G149C V51H K55H) using structural models.	200
Table 5.2 Glt _{Ph} protein yield for each variant and the calculated spin labelling efficiency	203
Table 5.3 Best fit simulation values for LT X-band cw-EPR spectra of Glt _{Ph} CM7 spin labelled with MTSL and/or Cu ²⁺ -NTA with reference to Cu ²⁺ -NTA in buffer	215
Table A1 A summary of the dissociation constants for Glt _{Tk} and Glt _{Ph} with either L-aspartate or L-glutamate.	290
Table A2 Best fit simulation values for LT X-band cw-EPR spectra of Glt _{Tk} S281C in detergent micelles	299
Table A3 Best fit simulation values for LT X-band cw-EPR spectra of Glt _{Tk} S281C in liposomes.	300
Table A4 Best fit simulation values for LT X-band cw-EPR spectra of Glt _{Tk} S281C in SMALPs.....	301
Table A5 Best fit simulation values for LT X-band cw-EPR spectra of Glt _{Tk} S281C in nanodiscs.....	302
Table A6 Best fit simulation values for LT X-band cw-EPR spectra of Glt _{Tk} D298C in detergent micelles	303
Table A7 Best fit simulation values for LT X-band cw-EPR spectra of Glt _{Tk} D298C in liposomes	304
Table A8 Best fit simulation values for LT X-band cw-EPR spectra of Glt _{Tk} I312C in detergent micelles	305
Table A9 Best fit simulation values for LT X-band cw-EPR spectra of Glt _{Tk} I312C in liposomes	306

Table A10 Best fit simulation values for LT X-band cw-EPR spectra of Glt _{Tk} M314C in detergent micelles	308
Table A11 Best fit simulation values for LT X-band cw-EPR spectra of Glt _{Tk} M314C in liposomes	309
Table A12 Best fit simulation values for LT X-band cw-EPR spectra of Glt _{Tk} H334C in detergent micelles	310
Table A13 Best fit simulation values for LT X-band cw-EPR spectra of Glt _{Tk} H334C in liposomes	311
Table A14 Best fit simulation values for LT X-band cw-EPR spectra of Glt _{Tk} A367C in detergent micelles.	312
Table A15 Best fit simulation values for LT X-band cw-EPR spectra of Glt _{Tk} A367C in liposomes.	313
Table A16 Best fit simulation values for LT X-band cw-EPR spectra of Glt _{Tk} A367C in SMALPs.....	314
Table A17 Results for MMM analysis of Glt _{Ph} variants spin labelled with MTSL	320
Table A18 Results for MMM analysis of Glt _{Ph} variants spin labelled with Cu ²⁺ -NTA	321
Table A19 Results for MMM analysis of Glt _{Ph} variants spin labelled with Cu ²⁺ -NTA and MTSL for inter-protomer distances.....	322
Table A20 Results for MMM analysis of Glt _{Ph} variants spin labelled with Cu ²⁺ -NTA and MTSL for intra-protomer distances.....	323

Glossary of terms

Å	Angstrom
ABC	ATP-binding cassette
ABC transporter	ATP-binding cassette transporter
AEBSF	4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride
ATP	Adenosine triphosphate
B ₀	Applied magnetic field
B _{eff}	Effective magnetic field
B _{local}	Local magnetic field
CM10	Glt _{ph} variant C321S G351C K132H D136H
CM11	Glt _{ph} variant C321S R52C A364H E368H
CM3	Glt _{ph} variant C321S G280C F50H L54H
CM7	Glt _{ph} variant C321S G149C V51H K55H
CM8	Glt _{ph} variant C321S T375C V51H K55H
CNS	Central nervous system
cryo-EM	Cryogenic electron microscopy
cw	Continuous wave
DDM	n-Dodecyl β-D-maltoside
DEER	Double electro-electron resonance
DL-TBOA	DL- <i>threo</i> -β-Benzyloxyaspartic
DMSO	Dimethyl sulfoxide
DOPC	1,2-dioleoyl-sn-glycero-3-phosphocholine
DPPH	2,2-diphenyl-1-picrylhydrazyl radical
DTT	Dithioreitol
EAAT	Excitatory amino acid transporter
EDTA	Ethylenediaminetetraacetic acid
EEl	Electron-electron interaction
eL4	Extracellular loop 4
EPR	Electron paramagnetic resonance
ESSEM	Electron spin echo-envelope modulation

EZI	Electron Zeeman interaction
FID	Free induction decay
FSE	Field swept echo
Glt _{Ph}	Glutamate transporter from <i>Pyrococcus horikoshii</i>
Glt _{Tk}	Glutamate transporter from <i>Thermococcus kodakarensis</i>
HFI	Hyperfine interaction
HP	Helical hairpin
HS-AFM	High-speed atomic force microscopy
iDEER	Inversion recover-filtered DEER
IF	Inward facing
IMAC	Immobilised metal affinity chromatography
ITC	Isothermal titration calorimetry
KPi	Potassium phosphate
L-Asp	L-aspartate
LB	Lysogeny broth
LFER	Linear free-energy relationship
LMV	Large multilamellar vesicles
LT	Low temperature
LUV	Large unilamellar vesicle
Lwpp	Linewidth peak to peak
M ₀	Bulk magnetisation
MD	Molecular dynamics
MFS	Major facilitator superfamily
MMM	Multiscale molecular modelling
MSP2N2	Membrane scaffold protein 2N2
MTSL	(1-Oxyl-2,2,5,5-tetramethyl-Δ3-pyrroline-3-methyl) Methanethiosulfonate
MW	Molecular weight
NMR	Nuclear magnetic resonance
NOE	Nuclear Overhauser effect
NQI	Nuclear quadrupole interaction

NSS	Neurotransmitter sodium symporter
PAG	Phosphate-activated glutaminase
NTA	Nitrilotriacetic acid
NTD	N-terminal domain
NZI	Nuclear Zeeman interaction
OF	Outward facing
PDB	Protein Data Bank
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PL	proteoliposome
PMSF	Phenylmethanesulfonyl fluoride
POPC	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
REFINE	Relaxation-filtered hyperfine
RF	radiofrequency
RF	radiofrequency
RIDME	Relaxation-induced dipolar modulation enhancement
RMSD	Root mean squared deviation
RT	Room temperature
SAXS	Small angle x-ray scattering
SBDD	Structure-based drug design
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SDSL	Site directed spin labelling
SL	Spin label
SLC	Solute carrier
SMA	Styrene maleic acid
SMALP	Styrene maleic acid lipid particles
smFRET	Small molecule fluorescence energy transfer
ss-NMR	Solid-state nuclear magnetic resonance
STD NMR	Saturation transfer difference nuclear magnetic resonance
Synbodies	Synthetic nanobodies
T ₁	Spin-lattice relaxation time

T ₂	Spin-spin relaxation time
TBOA	DL- <i>threo</i> -β-Benzoyloxyaspartic acid
TCDB	Transporter classification database
TEM	Transmission electron microscopy
TEMPO	2,2,6,6-tetramethylpiperidiny-1-oxyl radical
T _F	Filter time
TM	transmembrane
WT	Wild type
ZFS	Zero field splitting
τ _c	Rotational correlation time

1 Introduction

1.1 What are membrane transporter proteins?

Biological membranes are physical barriers controlling the interaction between cells or organelles and their surroundings. Separating the environment either side into chemically and physically distinct compartments allows control of the transport of substances across the lipid bilayer. The impermeability of the lipid bilayer to large polar or charged molecules means only small polar and uncharged molecules are able to pass through via passive diffusion.^{1,2} For substances unable to pass through the membrane via diffusion, membrane transporter proteins can be used to facilitate the movement across the lipid bilayer. Membrane transporters are either partially (peripheral) or fully embedded (integral) within the lipid bilayer, working to transport substances such as nutrients, ions, metabolites, drugs, and signalling molecules to maintain vital biochemical processes.¹⁻³ A large proportion of each membrane protein is in the hydrophobic environment of the lipid bilayer, with parts of the protein exposed to the intra- and extra-cellular hydrophilic environment.

The transporter classification database (TCDB) is a system which classifies membrane proteins according to their functional and phylogenetic information such as super-family, sub-family and substrate.⁴ Several other databases have been developed to represent transporters in restricted groups of organisms or certain categories of transporter such as solute carrier (SLC) tables,⁵ ABCdb⁶ and TransportDB.⁷ The main types of membrane transporter proteins are primary active transporters, secondary active transporters, channels, and facilitators (Figure 1.1). Primary active membrane proteins use adenosine triphosphate (ATP) to power the transport of substrate molecules against their concentration gradients e.g. ATP-binding cassette (ABC) transporters and ion pumps. Secondary active transporters facilitate the movement of a solute molecule against the concentration gradient and are subdivided into symporters and antiporters. The transport of the substrate molecule against its concentration gradient is driven by the co-transport of ions along

their electrochemical gradient. The difference is whether the two substrates are transported in the same direction (symport) or in the opposite direction (antiport). Channel proteins are open to both sides of the membrane allowing the passage of molecules, water, and ions down the concentration gradient, some of which control access of specific substances via gating mechanisms. Facilitator membrane proteins (also known as uniporters) use the passive transport of a single substrate down their concentration gradient.^{1,2}

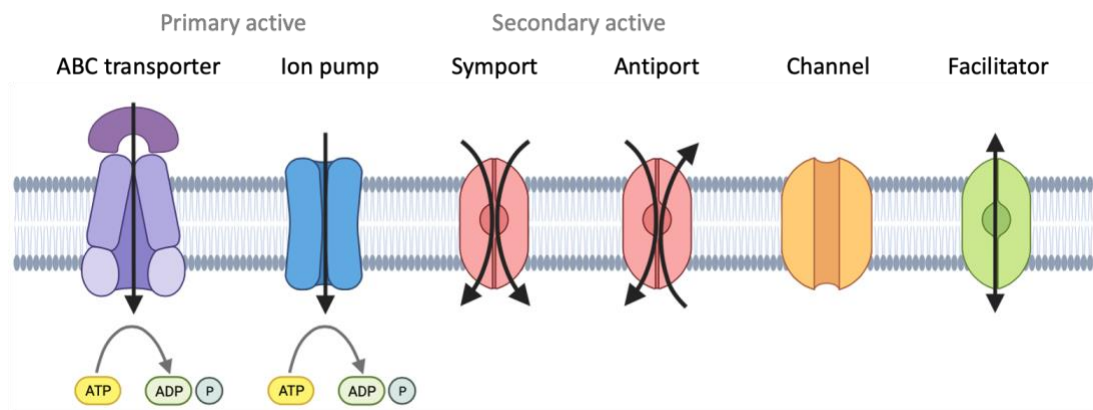


Figure 1.1 Schematic representation of the different types of membrane transporter proteins. These include primary active transporters (purple and blue), secondary active transporters (red), channel proteins (orange) and facilitators (green). The direction of transport is shown by black arrows, and ATP hydrolysis is shown where used. Figure created using biorender.com.

Membrane proteins account for one third of all proteins made by prokaryotic and eukaryotic organisms.^{8–10} Their role in controlling the transport of substances into and out of the cell is vital in maintaining essential processes and are often implicated in various disease pathways. Membrane proteins account for approximately 60% of therapeutic drug targets.¹¹ A major motivation for understanding structural changes and function of these proteins is to aid in the design and development of novel therapeutics.

1.2 Studying membrane transporter proteins

1.2.1 Membrane mimetics

To advance knowledge of membrane protein structure and function, biochemical and biophysical techniques need to be employed which requires several considerations. During the protein production and purification process a hydrophobic environment needs to be maintained to ensure the protein remains stable and functional. This is often achieved through the use of detergent micelles; however, this does not mimic a native membrane environment. Often biophysical and biochemical techniques require high-quality, functional protein samples in large quantities. Developments are continuously made to improve methodology, but the difficulties faced with low expression and purification yields, poor solubility and protein instability persist.^{12,13} These challenges help account for the small fraction of membrane proteins in the protein data bank (PDB) compared to soluble proteins.

To achieve more native-like membrane environments, alternatives to detergents are often used. These include liposomes, membrane scaffold protein nanodiscs and styrene maleic acid lipid particles (SMALPs) as shown in Figure 1.2. The native-like environment can aid protein stability and reveal different conformations to those observed in detergent micelles. In detergent micelles the hydrophobic aliphatic chain of the detergent molecule interacts with the protein itself, with the large hydrophilic head portion on the outside to ensure water solubility in aqueous buffer solutions. This results in the formation of a detergent micelle where the native lipids are removed and replaced with the detergent molecules to create a hydrophobic environment. A balance must be reached between solubilisation (yield) and structure preservation when selecting the detergent, in addition to the cost considerations, and will vary on a protein to protein basis.^{13,14}

Detergent-purified proteins can be reconstituted into liposomes which have an aqueous lumen separated from the external solution by a lipid bilayer where the protein is inserted. The size of the liposome, which in turn would affect the curvature,

is selected using extrusion with filters of a selected size. Like detergent, the lipid composition can be optimised to achieve maximum reconstitution.¹³ One of the main disadvantages of liposomes is the lack of control of the orientation of the protein or the amount of protein inserted per liposome which varies between proteins.¹⁵ Liposomes are the main reconstitution system if a separate internal and external environment is required, however this still requires prior purification of the protein into detergent.

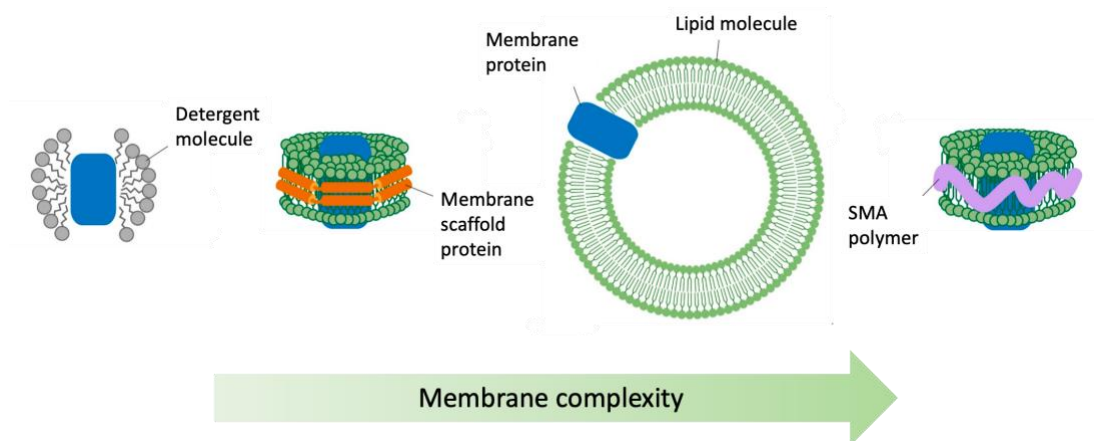


Figure 1.2 Membrane mimetics for structural and functional studies shown in increasing order of membrane complexity. Left to right: Detergent micelle, membrane scaffold protein (MSP) based nanodisc, liposome, and SMALP (polymer based nanodisc). Figure adapted from Yeh et al.¹⁶

Similarly, detergent purified protein can be reconstituted into nanodiscs which are frequently used in structural studies. Here the protein is inserted into a lipid bilayer in a disc-like structure with a homogeneous particle distribution and access to both sides. The lipid bilayer is encircled by a belt of two molecules of membrane scaffold protein (MSP) to hold the structure together, avoiding the orientational uncertainty in liposomes.^{13,17} The ratio of MSP and lipids with the protein, and the size of the MSP, needs to be optimised for each protein. Nanodiscs are generally more stable and offer the lipid bilayer arrangement like the native membrane. However, the lipids used do not replicate those of the native lipid bilayer which could affect protein interactions.

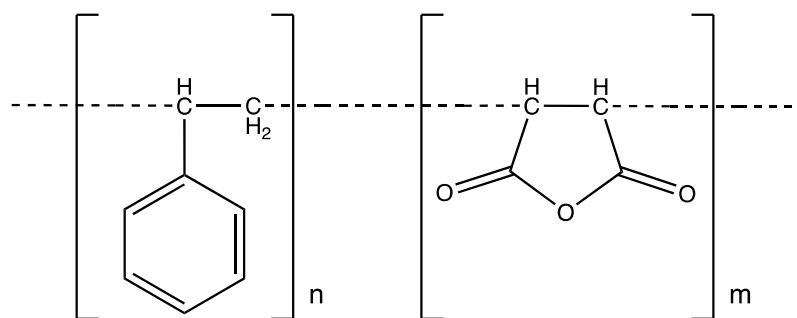


Figure 1.3 The structure of styrene maleic acid (SMA) polymer

SMALPs are an attempt to rectify the identified issue of lipid composition of nanodiscs. The SMALP reconstitution process uses styrene maleic acid (SMA) polymer in different ratios of styrene to maleic acid – most commonly 2:1 and 3:1 (Figure 1.3).^{18,19} It has been compared to a ‘cookie cutter’ where the SMA polymer forms a belt around the protein with the surrounding native lipids to generate particles similar to those of nanodiscs. The membrane protein and surrounding lipids are directly extracted from membrane vesicles into the SMALPs, removing the need for detergent solubilisation, unlike nanodiscs and liposomes.^{17,19} One particular advantage of SMALPs, in addition to the native lipids, is the increased functional thermostability observed for SMALP solubilised proteins compared to those in detergent micelles.^{20,21} However, there is a limited pH window before precipitation occurs, and there is a sensitivity to divalent cations²². Previous studies have also suggested the lateral pressure of the SMALP may limit protein conformational changes.¹⁸ Large protein complexes may not be compatible with SMALPs due to the small size of the disc, however alternative polymers are under development based on the same methodology. SMALPs are very much a new field with considerable potential in the future.

1.2.2 Complementary methods for studying membrane transporter proteins

Three of the most common structural biology techniques used to study membrane proteins are X-ray crystallography, cryogenic electron microscopy (cryo-EM) and nuclear magnetic resonance (NMR) spectroscopy. These techniques provide a snapshot to reveal 3-dimensional structures which are available in the protein data

bank (PDB). However, to gain a deeper understanding of the dynamics of the protein and intermediate conformational states other complementary techniques are often used which include electron paramagnetic resonance (EPR) spectroscopy and Förster (or fluorescence) resonance energy transfer (FRET). Revealing information about the structure and conformational changes at a molecular level is integral for understanding the associated mechanisms of transport. This knowledge is vital to aid in the development of effective therapeutics through structure based drug design (SBDD). Each of these techniques has the associated challenges of working with membrane proteins such as low yield and maintaining protein function after extraction from the native membrane but they also have their own associated limitations.^{10,12,13}

The majority of structures in the PDB were determined using X-ray crystallography, however only a small fraction of these are membrane proteins (<3%). In order to successfully solve the structure of a protein it must first be purified and crystallised before being subjected to an intense X-ray beam. The resultant diffraction pattern is used to obtain an electron density map which gives the location of each atom and in turn an atomic-resolution structure is determined.^{10,23} However, this requires high-quality crystals with uniform alignment of molecules within the lattice, which is difficult to achieve for membrane proteins. In addition, flexible regions of the protein are particularly difficult to solve and may not be visible in the electron density maps which is probably the most interesting part of a transporter.¹⁰

One of the most recent techniques in the structural biology toolkit is cryo-EM. This is particularly useful for proteins which are hard to crystallise. Thousands of single particles are imaged in a thin layer of non-crystalline ice using a powerful electron beam to produce a magnified image. The sample is rotated at multiple angles to build a 3-dimensional electron density map which is used to create a model of the protein.^{10,24,25} Near-atomic resolution can be achieved, however it is still difficult to resolve flexible or dynamic parts of the protein. Cryo-EM has a less stringent sample requirement because an ensemble of conformations can be studied, compared to the

uniformity needed in X-ray crystallography, but still requires meticulous protein preparation. It is also difficult to see short-lived intermediates.^{10,25}

Another structural biology method is solution state NMR, where examples of solved structures are in the PDB. Purified protein is placed in a strong magnetic field and probed with radio waves. The resultant spectrum reveals information about local conformations and distances between close atoms (based on resonances), which together with the primary sequence is used to build a 3-dimensional model.^{10,26} This technique commonly uses isotopic labelling, which is expensive, and adds an additional step into protein preparation. Solution state NMR is a better suited technique for flexible and dynamic residues and can also be performed in more native environments than X-ray crystallography and cryo-EM but it is restricted to small and medium proteins.²⁶ This makes it particularly challenging to study large membrane transporter complexes.

Unlike the structural methods, FRET is used to reveal kinetic parameters and overall mechanistic transitions with their associated time scales. The protein of interest needs to be labelled with two different fluorophores which typically bind to cysteine residues (so the functional integrity of the protein must be checked). One fluorophore acts as a donor; when emitted the energy transfers to the acceptor fluorophore which when in close proximity will fluoresce.²⁷ FRET is used to measure distance distributions between the donor and acceptor between 2–10 nm, on a ns to minute time scale. This technique is particularly useful for time-resolved measurements and single-molecule experiments.^{10,27}

Double electron-electron resonance (DEER) spectroscopy is an EPR technique which measures distances between two labels, offering complementary information to FRET. An advantage of the spin labels used in EPR is they are smaller than the fluorophores in FRET so are less likely to cause structural perturbation.²⁸ Both techniques are not limited by protein size and can be applied for proteins in different environments such as liposomes and nanodiscs to give insight into conformational dynamics. EPR has a higher sensitivity allowing minor populations to be observed,

and small distance changes with moderate resolution.^{12,29} This yields more accurate changes in the structural rearrangements of the protein compared to FRET. Different EPR techniques can be used to measure various properties such as distances between spin-labels and the local environment of the spin label which helps elucidate the associated mechanism of transport. Previous studies have found new intermediate states which have not previously been observed using other methods.^{12,29,30} However, it works best with a proposed structure or molecular model to aid interpretation of the information obtained.

1.3 EPR spectroscopy of secondary active membrane transporters

This chapter will go on to review some examples of applications of both continuous wave (cw) and pulsed EPR methods to investigate membrane proteins. A particular focus has been placed on secondary active symporters since both proteins studied in this thesis belong to this group of transporters. EPR studies mostly focused on the local environments of spin labels attached to proteins, and the distance changes between two or more spin labels on addition of ions and/or substrate.

EPR spectroscopy of PutP

The high-affinity proline transporter from *Escherichia coli*, PutP, is a secondary active transporter which utilises Na⁺ gradient to power movement of proline across the membrane. PutP is composed of 13 transmembrane domains (TMs), with the N-terminus in the periplasmic environment and the C-terminus in the cytoplasmic region. It has an important role in human infectious diseases and has been the subject of several EPR studies, all of which utilised spin-labelled cysteine residues to gain information on protein structure and dynamics.

The first EPR spectroscopy study was in 2000 where several different cysteine residues were placed in selected regions on TM2, TM3 and the loop connecting them.³¹ Limited information was available about the structure of PutP, with most information coming from membrane topology studies of similar proteins. The effect

of oxygen (concentrated in the membrane) and chromium oxalate (restricted to aqueous environments) on the relaxation time of the spin label were used to determine the position of the various residues relative to the lipid bilayer. Overall results of these studies supported the proposed structural model of PutP by identifying the specific residues within TM2 and TM3, in addition to identifying residues involved in the cytoplasmic loop. It was also found that upon addition of Na⁺ and/or proline, changes spin label mobility were observed for 2 of the 16 variants.³¹ These results suggested the conformational changes needed for substrate transport involved movement of TM2 and the cytoplasmic loop, with no change observed for TM3.

In 2004, the second PutP EPR spectroscopic study was published using three doubly spin-labelled variants. Four-pulse double electron-electron resonance (DEER) spectroscopy was employed for distance determination between residues to validate the proposed tertiary structure.³² Results again confirmed ligand-induced structural changes through changes in the distance distributions of PutP variants encapsulated in proteoliposomes. The changes in distance distributions observed further identified residue locations with pairs on opposite sides of the membrane exhibiting longer distance distributions than pairs of residues on cytoplasmic loops. Similarly, DEER studies were used to scan the shape of 16 double mutants located on TM9.³³ The resultant distance distributions were paired with simulations to create a helix-loop-helix model of the backbone structure of TM9 on PutP. A kink in TM9 was identified and proposed to be a hinge for the inward-facing structure to control substrate binding and release.

The Identification of other similar proteins was used to construct the first homology model of PutP, and further functional analysis led to the identification of possible Na⁺ and substrate binding sites.³⁴ In the leucine transporter from *Aquifex aeolicus*, LeuT, extracellular loop 4 (eL4) was shown to occlude the extracellular vestibule and also formed a thick gate in the benzyl-hydantoin transporter from *Microbacterium liquefaciens*, Mhp1.^{35,36} The gating function of eL4 with respect to the alternating access model of secondary active transport was further investigated using EPR

spectroscopic studies of PutP. The first study used singly labelled PutP variants in liposomes to investigate structure and orientation of eL4 upon ligand binding based on side chain mobility, polarity and accessibility of paramagnetic quenchers– in addition to distance measurements.³⁷ The results revealed the division of eL4 into two α -helical segments, identifying the residues in each, which form the extracellular gate. Ligand induced conformational changes were found to be transmitted via Phe314 which anchors eL4 to the core of the transporter via hydrophobic interactions with TM1. After substrate binding, a pulling force from amino acid interactions above the binding sites is proposed to cause inward movement of eL4, closing the extracellular gate.

A second study confirmed eL4 was the extracellular gate of PutP using DEER spectroscopy.³⁸ A specific focus on eL4 and the outer region of TM10 was used to investigate the alternating access mechanism and further the understanding of eL4 functional significance. Substitutions of Glu311 and Ala404 did not significantly impair binding of substrate or sodium ions, but they did cause a more open conformation compared to WT. The interactions between Glu311 at the tip of eL4 with the peptide backbone of TM10 were found to stabilise the inward conformation with external gate closing.³⁸

EPR spectroscopy of LeuT

Unlike PutP, the structure of the leucine transporter, LeuT, had been solved prior to EPR studies. Crystal structures revealed a 5+5 inverted repeat of transmembrane domains which are also referred to as a 'LeuT fold' and have been observed in other secondary active transporters.^{39,40} LeuT is composed of 12 TMs in total, with extracellular and intracellular loops. Based on structural information, transport of the primary substrate leucine was proposed to occur via an alternating access mechanism, where transport is powered by the electrochemical gradient of co-transported Na⁺ ions. Claxton et al. used EPR spectroscopy to investigate the associated conformational changes and structural dynamics for substrate transport.³⁵ A combination of EPR techniques were used to study 41 single and 6 double cysteine

mutants with a focus on spin-label mobility, accessibility and distance determination using pulsed EPR (DEER) spectroscopy. The cysteine residues were inserted in regions to focus on the extracellular vestibule associated with ion and substrate binding. Results identified Na⁺ dependent conformational changes where Na⁺ binding formed a dynamic intermediate outward-facing state involving the movement of eL4. This conformation exhibited increased accessibility of the substrate binding site to the extracellular environment ready for substrate binding. Upon addition of leucine, this accessibility was reduced through both local and global conformational changes as the protein transitioned to an occluded conformation.³⁵

To further elucidate the transport mechanism, research into the region of the protein exposed to the intracellular environment was needed. A second EPR study used DEER to further build on knowledge surrounding Na⁺ and substrate dependent conformational changes, in addition to identifying structural motifs in the transport mechanism and investigate isomerisation between the different conformations previously identified in crystal structures.⁴¹ A variety of cysteine variants were used to target both the intracellular side (on TM4 and TM9) and extracellular side (on TM3 and TM5) of LeuT. In an apo state, LeuT was shown to be in a mixture of conformations (open and occluded) and to isomerise between them. Addition of Na⁺ favoured the outward-open conformation as observed in the previous EPR study, in addition to an inward closed conformation, showing the structural rearrangements were mechanistically important.⁴¹ Previous studies found the leucine dependent coupling of the extracellular and intracellular side of LeuT involved TM6, but this study had the novel addition of TM7. Movement of TM7 had not previously been seen in crystal structures which was likely to be a result of the typically outward facing conformations. As with the previous EPR study, addition of leucine results in an occluded conformation where the binding sites were closed to the external environments.^{35,41}

Overall, this study resulted in the proposition of a new mechanism (Figure 1.4), deviating from the rocking bundle proposed based on crystal structures. EPR studies showed the entire transport domain rotated relative to the scaffold domain, with

TM1, TM6 and TM7 acting as hinge points around which the domains moved. The model also included eL4 movement which had not previously been considered and is thought to act as a gate.⁴¹

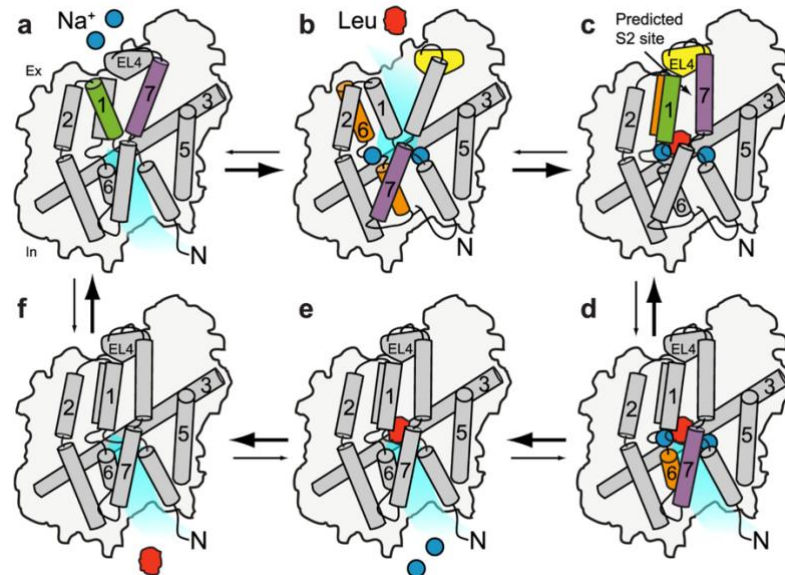


Figure 1.4 The proposed transport model of LeuT based on EPR spectroscopic data. The alternating access mechanism shows LeuT transitioning between outward facing (top) and inward facing (bottom) conformations upon binding of Na^+ (blue) and leucine (red). Transmembrane helices involved in each step are shown in colour. The cycle begins (a) with apo LeuT in an inward facing conformation, which transitions to an outward facing conformation (b) upon binding two Na^+ ions and the intracellular gate which closes. Substrate (leucine) binding causes an outward facing occluded state (c). Transmembrane helices move to transition to an inward facing state which opens (d). The two sodium ions are released (e) followed by substrate (f), and the cycle (a-f) resets moving through inward and outward facing isomerisation. Figure from Kazmier et al. 2014.⁴¹

EPR spectroscopy of vSGLT

The Na^+ dependent galactose transporter from *Vibrio parahaemolyticus* (vSGLT) has the same 5+5 inverted repeat as LeuT and is also a secondary active transporter proposed to operate via an alternating access mechanism illustrated in Figure 1.5. Previous crystal structures revealed inward open and inward occluded conformations.^{42,43} Despite these similarities, DEER studies revealed mechanistic differences to LeuT⁴⁴. The preference for inward facing conformations was confirmed using DEER spectroscopy of four single cysteine mutants of the dimeric protein. One

labelling site was located on each of TM2, TM3, TM6 and TM7 covering both the intracellular and extracellular side of the protein. There are no crystal structures representing an outward facing conformation of vSGLT, only models based on a similar system (SiaT).⁴⁴ There were small global and local rearrangements moving from apo to Na⁺ bound vSGLT. DEER distances for galactose-bound vSGLT showed a shift in equilibrium toward outward open and outward occluded conformations, correlating with proposed models.⁴⁴ These results contrast to LeuT, where in the presence of Na⁺ an outward facing conformation is preferred. This could be due to the different ion: substrate stoichiometry where LeuT has a 2:1 ratio and vSGLT 1:1.^{35,41,44} It was proposed that under physiological conditions with an external Na⁺ concentration, the presence of both membrane potentials and native lipids, an outward conformation may be stabilised upon binding Na⁺.

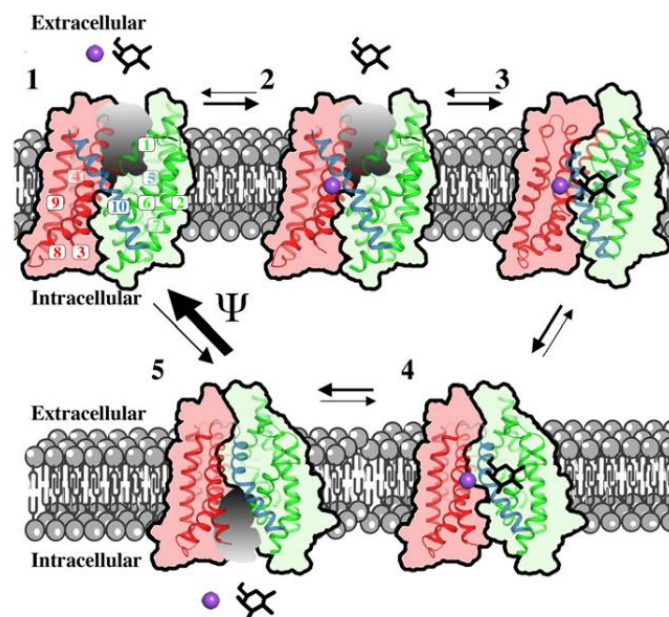


Figure 1.5 The proposed sugar transport mechanism of vSGLT. The protein is proposed to mainly occupy an inward facing conformation (5) with isomerisation to outward-facing states(1) under physiological conditions driven by the membrane potential (Ψ). Sodium ion binding to the outward facing state (2) is followed by substrate (sugar) binding, and then becomes an outward facing occluded conformation (3). The protein isomerises to an inward facing state (4), releasing sugar and sodium ions to the intracellular environment. The protein is returned to the apo, inward-facing conformation (5). Transport is proposed to occur via a rocking-bundle mechanism which differs to that of structurally similar LeuT. Each protomer is shown separately, with sodium shown in purple and substrate in black. Figure from Paz et al. 2018.⁴⁴

EPR spectroscopy of Mhp1

Mhp1 is a Benzyl-hydantoin transporter from *Microbacterium liquefaciens* - also a secondary active membrane transporter proposed to operate via an alternating access mechanism. Crystal structures in both inward and outward facing conformations found the 'LeuT fold' or 5 helix inverted repeat like LeuT and vSGLT despite only sharing a 15% and 16% sequence identity respectively.^{36,45} EPR studies investigating the conformational dynamics during the transport cycle, and coupling to ion gradients found a different mechanism of transport to LeuT, highlighting that proteins with the same structure or fold do not necessarily function in the same way.^{41,46} The only EPR study for Mhp1 used DEER spectroscopy to measure distance changes on both the intracellular and extracellular facing regions upon addition of Na⁺ and substrate (Figure 1.6).

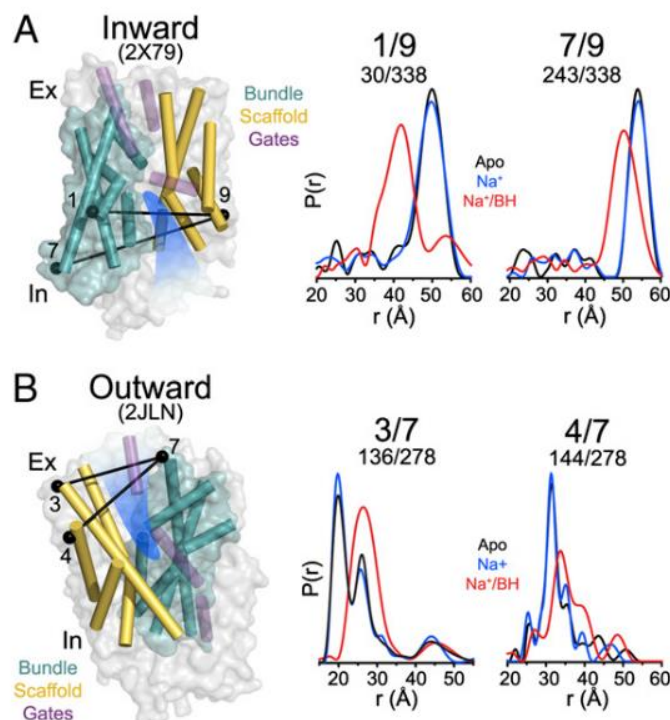


Figure 1.6 Investigation of conformational changes of Mhp1 on addition of sodium and substrate. Distance distributions were calculated to report conformational changes on both the intracellular (A) and extracellular (B) sides of the protein. Figure from Kazmier et al 2014.⁴⁶

Results showed that crystal structures represented intermediate inward and outward facing states of Mhp1 in solution and corroborated proposed transport models based on the structural data. Interestingly, binding of Na^+ did not change the energetics/binding equilibrium between the inward and outward facing state seen for apo Mhp1. This contrasted to LeuT where Na^+ binding favoured an outward open conformation. The driving force for transitions in the transport cycle of Mhp1 was observed during substrate binding (in the presence of Na^+). This led to the formation of an occluded conformation via ligand dependent movement of the eL4 gates.⁴⁶ Like vSGLT the ion substrate stoichiometry was 1:1 compared to 2:1 for LeuT, which could account for some of the diverse structural rearrangements seen in crystal structures. Overall, this EPR study suggested Mhp1 operates via a different mechanism to LeuT where no ion-dependent conformational changes are observed.

EPR spectroscopy of BetP

Another example of a secondary active transporter which has been studied by EPR spectroscopy is the betaine transporter from *Corynebacterium glutamicum*, BetP. It is an uptake system activated by hyperosmotic stress where substrate transport is powered by symport of Na^+ . As with other secondary active transporters, the homotrimeric BetP is proposed to operate via an alternating access mechanism.^{47,48} The inactive and activated states were investigated using continuous wave (cw) EPR spectroscopy and distance determination (DEER) studies to elucidate conformational changes upon activation.⁴⁹ Cysteine variants were designed to specifically focus on the C-terminal domain or were located in close vicinity to focus on the dynamics in this region which is proposed to play a key role in activation. A single cysteine variant was created at the beginning of the C-terminal domain, at the end and a double mutant combining the two. Addition of Na^+ and substrate were found to cause no change in the immediate spin label environment, as reflected in very small line-shape changes. However, the distances in the core of the trimeric structure in liposomes were different to those observed in crystal structures giving experimental evidence of conformational change on activation, as shown in the distance changes in Figure

1.7. Reconstitution into liposomes showed an increased interaction of the C-terminus with adjacent structures which would be lipids or other BetP molecules.⁴⁹

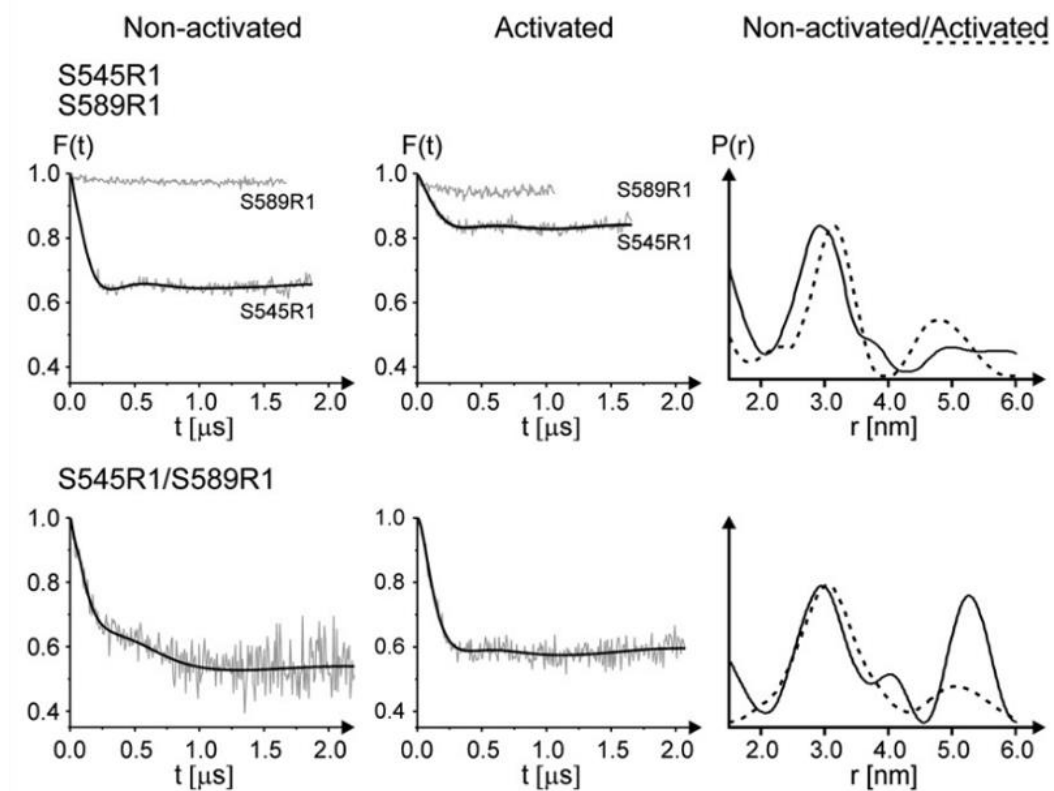


Figure 1.7 DEER studies of BetP using single and double cysteine variants under non-activated (solid line) and activated conditions (dashed line). R1 represents MTSL attachment. Activation occurred via addition of NaCl. Distance changes indicated conformational changes upon activation. Figure from Nicklisch et al. 2012.⁴⁹

A second study of BetP combined DEER spectroscopy with ensemble-biased meta dynamic molecular simulations to test the relevance of conformations predicted using distance distributions which did not agree with crystal structures.⁵⁰ Spin labels were attached to TM7 and TM8, either side of the extracellular pathway. Like vSGLT and Mhp1, Na⁺ bound BetP showed a preference for inward facing states. Following addition of substrate, BetP distance distributions did not correspond inward or outward open conformations and was proposed to be an occluded conformation, as observed for other similar proteins when substrate bound.

BetP was used as a test system to advance DEER spectroscopy for membrane proteins. The accessible distance distribution is shorter for detergent solubilised and lipid-bound membrane proteins due to the reduced coherence time of spins. Spindler et al. created a novel 7 pulse sequence to measure longer distances than the typical 4-pulse sequence.⁵¹ This was based on a scheme which recouples dipolar interactions to create a longer observation time window. For BetP this allowed distance measurement up to 6 nm which was not achieved using other methods.

EPR spectroscopy of GalP

GalP is a D-galactose H⁺ symporter from *Escherichia coli*, often studied as a homologue of the human glucose transporters. Prior to EPR studies GalP had not been crystallised but suggestions were made for a 12 TM helix assembly which is common for the secondary active transporters.⁵² The first EPR study was used to investigate GalP in native plasma membrane vesicles. A single labelling site with constrained mobility was used with different spin labels to measure the rotational diffusion. The selected cysteine residue was shown to block activity when chemically modified. Each of the three spin labels showed highly constrained environments which correlates with predictions of close proximity to the substrate binding site which could be sterically blocked by spin labels.⁵³ Overall the results of this study were consistent with the proposed 12 TM helix assembly, however less tightly arranged than predicted. This was corroborated by a second EPR study which involved spin-labelling of lipids, as opposed to cysteine residues on protein.⁵⁴ The aim of this study was to investigate the stoichiometry and selectivity of protein-lipid interactions, again in the native environment of the inner membrane. The stoichiometry informed on the assembly of TMs by the intramembranous perimeter, and the selectivity provided information about amino acids at the membrane-water interface. Spin-label motion was highly constrained, agreeing with the previous study, and led to the suggestion of a large hydrophobic surface interacting with the inner membrane lipids.^{53,54}

EPR spectroscopy of LacY

Unlike GalP, the structure of lactose permease from *Escherichia coli*, LacY was solved in inward open and occluded states prior to EPR studies. LacY is a secondary active sugar and H⁺ symporter composed of 12 TM helices organised into two 6 helix bundles.^{55,56} DEER studies were used to measure the conformational dynamics of LacY upon binding sugar for various double cysteine mutants. Cysteine residues were inserted at the ends of TM helices on both the periplasmic and cytoplasmic sides, in addition to a conformationally restricted C154G mutant.⁵⁷ Apo LacY distances supported model predictions made due to the absence of an outward facing crystal structure. Upon substrate binding, distance changes were observed with opposite movements either side of the membrane. An increase in distance was seen on the periplasmic side, and decreased distance on the cytoplasmic side which corresponded to an outward facing state. The observed distances ranged between 4–14 Å for the periplasmic side and 4–21 Å for the cytoplasmic side, supporting the alternating access mechanism by transitioning between inward and outward facing conformations. For C154G LacY, no changes were observed on the periplasmic side but opening on the periplasmic side was seen as with the other variants.⁵⁸ Overall rotational movements were proposed during conformational rearrangements which corroborate kinks and bends seen in crystal structures proposed to enable flexibility.

A second study was conducted to further investigate the structurally unsolved outward open state of LacY.⁵⁹ The distance changes using DEER spectroscopy for a specific residue were significantly different to LacY model simulations. This study tested of the effect of spin label size and internal flexibility on DEER distance measurements. Molecular dynamic dummy spin-label simulations showed the orientation and movement of spin-labels significantly impacts distance distributions and should be used for proper interpretation of spin-label distance distribution data of LacY.⁵⁹

EPR spectroscopy of PepT_{so}

The proton coupled oligopeptide transporters were studied using molecular dynamics, crystallography, functional assays and DEER spectroscopy to investigate transport dynamics.⁶⁰ DEER spectroscopy on the transporter from *Shewanella oniedensis* PepT_{so} was used to validate the outward open model for this group of transporters. PepT_{so} has two 6-helix inverted repeats and is a proton coupled symporter proposed to operate via an alternating access mechanism. Substrate transport is controlled by the presence of extracellular and intracellular gates identified in crystal structures.⁶¹ Eight pairs of cysteine residues were studied to determine 3 periplasmic distances and 5 cytoplasmic distances. The results revealed several different conformations for the highly dynamic protein, where all distances were within the bounds of predictions based on models. However, the outward open model of PepT_{so} did not closely correlate with the DEER distance distributions, particularly when compared to the DEER data and crystal structure for the inward open conformation. This was proposed to be the result of more than two conformations being present which complicated the data.⁶⁰ The overall mechanism for PepT_{so} is suggested to be 'scissor-like' with cooperative functioning between inverted topological repeats. 5 of the 8 variants studied were at equivalent positions to those in previous LacY EPR studies but the distance distributions obtained were very different.^{57,60} This further highlights that despite similar function or structures, proteins can operate via different mechanisms.

A review on published EPR studies of Glt_{ph} is included in Section 1.5.4 as part of the biophysical and biochemical studies of the glutamate transporters studied in this thesis.

Summary of EPR studies of secondary active transporters

Membrane proteins are fundamental for vital cellular processes, with their dysfunction linked to various disease states some of which can be fatal. Structural biology aids in solving the 3-dimensional structure of proteins, revealing different

conformational states and identifying structural similarities to other proteins. This advances understanding of protein mechanism and function which can be used to develop novel drugs and therapeutics for the implicated health disorders. Despite only revealing static snapshots, structural biology provides a strong starting point for spectroscopy and other techniques, as discussed in the review. The absence of a structure does not prevent the use of EPR spectroscopy, but it does make interpretation of results more challenging with the lack of a 3D structure or model. EPR spectroscopic techniques are used to reveal dynamic information about protein function and work to elucidate the dynamic transport mechanism through information on the spin-label environment and measuring distance distributions between two or more spin labels. This means EPR has the potential to reveal transient conformational states which have not been observed in static structures. Considerations of the locations of spin labels and the effect on protein structure and function need to be made, in addition to protein stability under the preparation and experimental conditions. Overall combining the use of structural biology methods, such as X-ray crystallography and cryo-EM, with EPR spectroscopy has proven to be a successful tool kit to study the structure and functional dynamics of macromolecular complexes.

1.4 Neurotransmission and the glutamate transporters

1.4.1 The SLC superfamily

The largest family of membrane transporter proteins are the solute carrier (SLC) superfamily which contains 66 sub-families, all of which use facilitative diffusion or secondary active transport.^{3,62} These are included in the SLC tables which are interconnected within the TCDB. The SLC gene nomenclature system follows the names SLCX, where X is a number representing the sub-families 1–66. To be classified as an SLC transporter, there must be at least 20% sequence identity to other members of the family. The SLC members transport a range of chemically diverse substances, with some being more specific than others. The vital role of the SLC superfamily is to transport nutrients, ions, metabolites, and signalling molecules across the cell

membrane. Dysfunction of these proteins is associated with various disorders, with 71 of the 287 SLC genes in the brain linked to neurological disorders.³ Sixty out of 66 SLC transporters families have members identified in the brain to perform various functions.^{3,63} These include energy metabolism, transport across the blood-brain barrier and regulation of neurotransmitter levels in the chemical synapse.

1.4.2 Neurotransmission in the central nervous system

The chemical synapse is the space between pre- and post-synaptic neurons, controlling communication between the two as well as to non-neuronal cells such as muscles. They connect neurons in the central nervous system using chemical and electrical signals. Neurotransmitters are packaged into membrane-bound synaptic vesicles in the pre-synaptic neuron and released into the synaptic cleft (by exocytosis). The release of neurotransmitter is stimulated by an action potential which induces a change in the membrane potential. The neurotransmitter diffuses across the synaptic cleft and binds to specific receptors (membrane proteins) on the post-synaptic neuron. This stimulates uptake of neurotransmitter into the post-synaptic neuron and terminates the signalling event. Neurotransmitter molecules are either reabsorbed by the pre-synaptic neuron and repackaged or metabolically broken down.^{64,65} This completes part of the circuit within the central nervous system and allows signal transduction to continue. An example of which is shown in Figure 1.8.

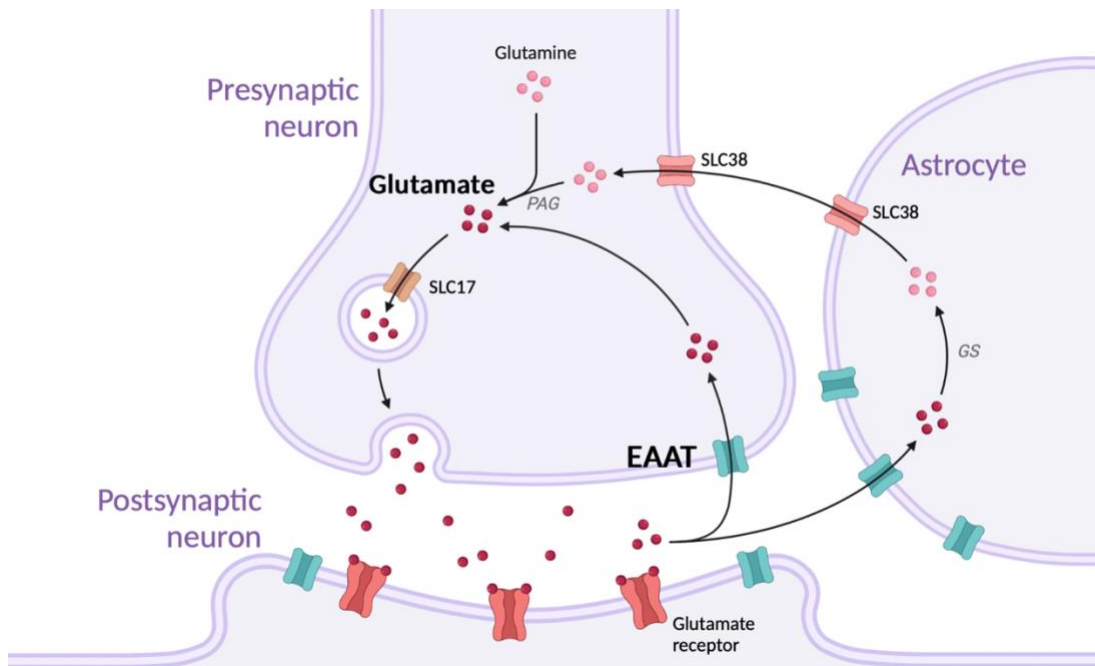


Figure 1.8 The glutamatergic synapse highlighting the role of the EAATs in glutamate regulation in the synaptic cleft. Glutamate (red circles) is converted to glutamine (pink circles) by glutamine synthetase (GS), and glutamine converted to glutamate by phosphate activated glutaminase (PAG). Figure made using biorender.com.

Glutamate is the most abundant neurotransmitter in the central nervous system and is involved in the glutamate-glutamine cycle (Figure 1.8). This, like all signal transduction events in the central nervous system, includes several cellular and molecular participants. The glutamatergic synapse is essential for learning and memory, and hence disruption of glutamate homeostasis can lead to various neurological conditions.^{66,67}

Glutamate is packaged into a synaptic vesicle in the presynaptic neuron by an SLC17 family member. When the membrane potential changes, glutamate is released into the synaptic cleft. This change in potential is caused by an influx of Ca^{2+} ions via gated calcium channels when nerve impulses are transmitted to the pre-synaptic neuron.^{3,68} Glutamate cannot be metabolised in the chemical synapse, so it has to be removed to terminate the signal and prevent excitotoxicity. Glutamate is transported either into the post-synaptic neuron or into astrocytes by excitatory amino acid transporters (EAATs) to maintain the extracellular concentration below excitotoxic levels. In astrocytes, glutamate is converted into glutamine by glutamine synthase

where it is then transported back to the pre-synaptic neuron via SLC38 neutral amino acid transporters, and also by EAATs.³ Glutamine is then converted back to glutamate by phosphate-activated glutaminase (PAG) to complete the glutamate-glutamine cycle, which starts again.⁶⁸

The glutamate receptors responsible for removing glutamate from the synaptic cleft are either ionotropic or metabotropic receptors. In ionotropic receptors, the binding of glutamate causes conformational changes and an influx of Na⁺ and/or Ca²⁺ (and efflux of K⁺) via voltage-gated cation channels. In turn this triggers depolarisation of the post-synaptic neuron and results in signal transmission. Slower-acting metabotropic receptors work differently to ionotropic receptors with a series of intracellular events but ultimately also result in signal transmission upon binding glutamate.^{65–67}

1.4.3 Excitatory amino acid transporters

The SLC1 family of transporters are high-affinity glutamate and neutral amino acid transporters responsible for terminating excitatory neurotransmission. Members of the SLC1 family include five EAATs and two alanine serine cysteine transporters (ASCT's), where the latter is responsible for neutral amino acid transport.⁶⁹ The five EAATs have the primary function of transporting glutamate and other ions (Na⁺, K⁺ and H⁺) across the membrane. EAATs 1 and 2 are located on astrocytes, with EAAT2 proposed to be the most abundant and responsible for 80% of glutamate reuptake activity.^{70,71} EAAT3 is found on the post-synaptic neuron, with EAAT4 specifically located in the post-synaptic neuron of Purkinje cells (in the cerebellum). The most unique member is EAAT5 which is located on the pre-synaptic neuron of cells in the retina. EAAT1–3 are also found in other places outside of the central nervous system such as the placenta and heart, but the majority are in the brain.^{70–72}

The numerical nomenclature of the EAATs was devised after three members were identified using their cDNA in 1992, where they were given the human gene names: SLC1A1, SLC1A2 and SLC1A3.^{73–75} These proteins were isolated from rat and rabbit

brains, and were subsequently cloned from human isoforms later where they were termed to EAAT3, EAAT2 and EAAT1 respectively.⁷⁶ SLC1A4–7 were then identified due to their close sequence homology to the previously cloned members where SLC6 is EAAT4 and SLC7 is EAAT5.^{77,78} Since their discovery, the EAATs have been linked to various neurological conditions including Alzheimer's disease, schizophrenia, epilepsy and ischemia.^{79–82} These are caused when an excess of glutamate builds up in the synaptic cleft, causing over-stimulation and glutamate signalling pathways to be triggered which can result in neuronal death. Understanding the EAATs, their locations and substrate/ion specificity aids the development of therapeutics and drugs to manage neurological disorders.⁷⁰

The first human EAAT crystal structure was published in 2017 (of EAAT1) using X-ray crystallography, 25 years after their initial identification using cDNA in 1992 for EAAT2.^{73,83} There are now human EAAT structures (EAATs 1–3) in the PDB which were solved using cryo-EM to reveal the architecture of the eukaryotic transporters^{84–88}. These include snapshots of the transporters in different binding states to elucidate the conformational changes the protein undergoes during the transport cycle. The recent structures solved using cryo-EM highlight the promise of emerging technologies to enable the study of human proteins, as opposed to using model systems, therefore, enabling advancements in the design of novel drugs and therapeutics. The EAAT structures exhibited high structural similarities to each other (Figure 1.9) and to the more widely studied archaeal homologues which will be discussed in Section 1.5.

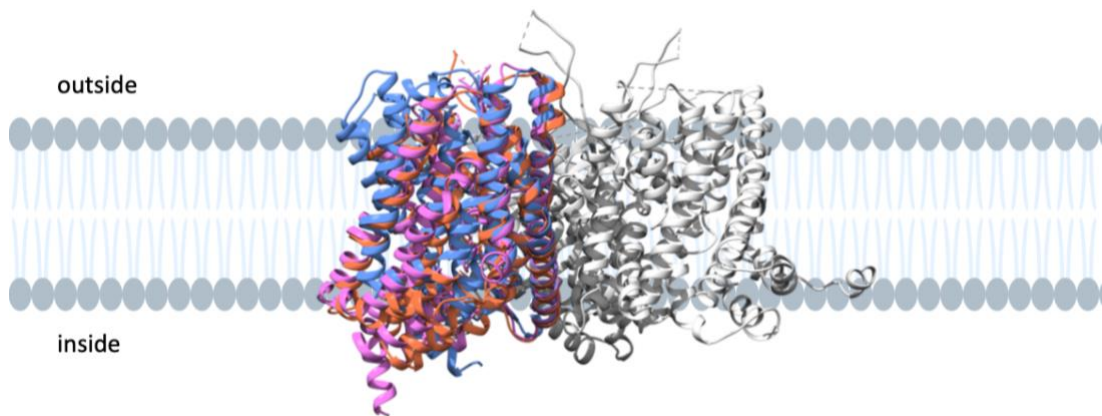


Figure 1.9 Cartoon representation of EAATs 1-3 in an inward facing conformation. A single protomer of EAAT1 (orange red, PDB 7NPW), EAAT2 (pink, PDB 7XR4) and EAAT3 (cornflower blue, PDB 8CV3) are overlaid to show the high structural similarities, with the other two protomers of EAAT1 shown in grey to highlight the trimeric structure.

Initial functional studies of the EAATs in rat and rabbit brains revealed Na^+ and K^+ dependencies.⁸⁹ Further studies confirmed substrate transport was a Na^+ -dependent process where glutamate transport across the membrane is associated with a large inward current.^{90,91} Voltage clamp studies with pH sensitive fluorescent dyes also found the co-transportation of a proton. Kinetic measurement under different ion and substrate concentrations revealed for each glutamate molecule transported, three Na^+ ions and one proton are co-transported, with counter transport of one K^+ ion.^{92,93} Glutamate transport is dependent on the binding and co-transport of these cations. Glutamate is transported up-hill against the concentration gradient, with downhill transport of the cations hence the EAATs are secondary active transporters. The uphill substrate transport is powered by the free energy from electrochemical potential gradients of the cations. The substrate: ion stoichiometry can support a 10^6 fold gradient of glutamate across the membrane. This maintains extracellular L-glutamate concentrations in the low nanomolar range.^{94,95} In addition to the three identified cations, the EAATs also transport chloride ions via a ligand-gated channel. Chloride transport is thermodynamically independent of glutamate transport but is Na^+ and K^+ dependent. This is proposed to counterbalance the positive ion influx and stabilises membrane potentials. It also aids in regulating synaptic transmission by

reducing glutamate release and mediates inhibitory currents to offset depolarisation from glutamate transport.^{96,97}

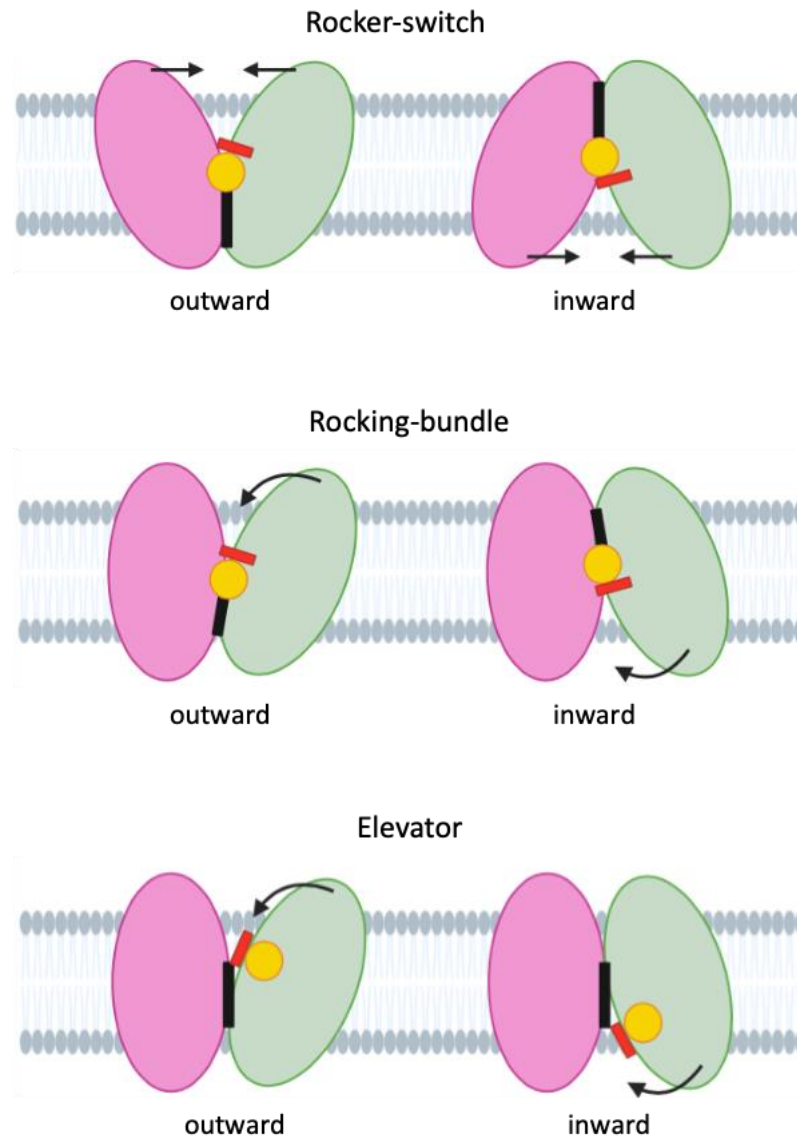


Figure 1.10 The three models of an alternating access mechanism for substrate transport across a membrane. Yellow circles represent substrate and ion binding sites, red rectangle represents gates, and the black rectangle represents the barrier between the two domains where each is represented by a pink or green oval. The movement of each is shown by black arrows. In the rocker-switch model the two domains move symmetrically to alternately expose the substrate binding site to either side of the membrane. In a rocking-bundle model, the two domains are different. One domain (pink) remains fixed, with the other domain (green) undergoes an asymmetric rearrangement around the binding site. Elevator mechanisms differ to the rocker-switch and rocking-bundle with the substrate binding site moving with the domain (green), relative to the other domain (pink) which remains fixed, as highlighted by the fixed barrier (black). Figure made using biorender.com.

An alternating access mechanism is the most common model for secondary active transporters, including the EAATs.⁹⁸ The protein transitions between inward-facing and outward-facing states to expose substrate and ion binding sites to either side of the membrane. The three main models of alternating access mechanisms are the rocker-switch, rocking-bundle, and elevator models (Figure 1.10).^{98,99} Proteins have been assigned to different models based on structures in multiple conformations, biochemical and biophysical analysis and from molecular dynamic calculations.

The rocker-switch mechanism involved two bundles moving symmetrically (i.e. 'rocking') around the central substrate-binding site, as used by the major facilitator superfamily (MFS) transporters.⁹⁹ This differs to a rocking-bundle model by the asymmetrical rearrangement around the central binding site. The two bundles are dissimilar and are termed scaffold and transport domains, where the transport domain moves relative to the fixed scaffold domain by a rocking movement. This has been observed for LeuT, an SLC6 homologue.¹⁰⁰ The final model is the elevator-type mechanism which also involves a transport and scaffold domain. The transport domain all moves relative to the scaffold domain as one rigid body, so unlike the other two models the substrate and ion binding sites do not remain fixed centrally.^{98,101}

An archaeal homologue of the EAATs was the first protein to exhibit the elevator mechanism which has been shown to apply to the EAAT members.^{101,102} To control access and release of substrates and ions into the binding sites, gating mechanisms are used. The EAATs (and their homologues) have a single gate mechanism however other proteins have been shown to have two gates.¹⁰¹ When the gate is open, the transport domain cannot move, preventing transport of the substrate. Most proteins which exhibit the elevator type mechanism are homodimers or homotrimers, with the level of cooperativity between protomers varying between different systems.¹⁰¹ The EAATs for example showed functional independence.^{103–107} As new studies are published, modifications are made to the specific details of the alternating access elevator mechanism. The most recent cryo-EM structures of EAAT3 have led to a comprehensive mechanistic proposal with details of interplay between conformations of conserved motifs, solute binding and gating movements.⁸⁴

Protein structures reveal some of the conformational changes present during the transport mechanism. Using dynamic studies, such as electron paramagnetic resonance spectroscopy, to add to the structural data enables detection of intermediate states that may not have been previously observed. The combination of structural, biochemical, and biophysical studies of the EAATs and archaeal homologues has allowed detailed transport mechanisms to be proposed. The main events that influence the kinetics of the alternating access mechanism are i) the rate of substrate binding; ii) the probability of transport and uptake efficiency; iii) how quickly glutamate is transported across the membrane and the protein returns back to the original state; and iv) the proximity of the transporter to the synapse – each of which would differ between the EAAT proteins and their associated functional capacities.^{67,71,94}

1.5 Glt_{Ph} and Glt_{Tk} – archaeal homologues of the mammalian EAATs

Prior to 2004, there were no crystal structures of the mammalian glutamate transporters or their homologues. The first published crystal structure was for the archaeal homologue glutamate transporter from *Pyrococcus horikoshii*, Glt_{Ph}.¹⁰⁸ In 2013, a crystal structure for a different archaeal homologue was published: glutamate transporter from *Thermococcus kodakarensis*, Glt_{Tk}.¹⁰⁹ These two transporters are used as model systems for the EAATs due to easier expression and purification compared to mammalian proteins, giving an insight into the structure-function relationships. Both proteins have been crystallised in a range of conformational states and binding conditions giving an invaluable insight into the proposed mechanism of transport. Glt_{Tk} and Glt_{Ph} have a 77% amino acid sequence identity to each other and 30–40% sequence identities with the EAATs with high conservation for residues in functionally important regions such as substrate and ion binding sites.^{83,108–112}

The main differences between the archaeal homologues and the EAATs are in the substrate affinities. The EAATs transport D/L-aspartate and L-glutamate in micromolar affinities, with D-glutamate transported in a lower millimolar affinity.^{72,76,78} For both

archaeal homologues the binding affinity for L-aspartate (nanomolar range) was higher than in the EAATs. Fluorescence studies of Glt_{ph} found comparable affinities for D/L-aspartate but only weak binding was observed for L-glutamate.¹¹¹ Studies using isothermal titration calorimetry (ITC) calculated comparable binding affinities for the two aspartate isomers with Glt_{Tk},¹¹³ like Glt_{ph}, however there is no published data for D/L-glutamate. In bacteria and archaea glutamate transporters are responsible for the uptake of glutamate and aspartate as nutrients, contrasting to the role in glutamate regulation in their mammalian equivalents.¹¹⁰ The archaeal homologues transport three Na⁺ ions per substrate molecule, eliminating the co-transport of one proton and counter transport of a K⁺ ion seen in the EAATs (Figure 1.11).^{102,114–116} However, the uncoupled chloride conductance is still present.^{115,117–119} Glt_{Tk} and Glt_{ph} have been proven to be informative model systems of the EAATs.⁸³ In this thesis, both systems have been studied using magnetic resonance techniques to explore conformational changes and their associated mechanisms.

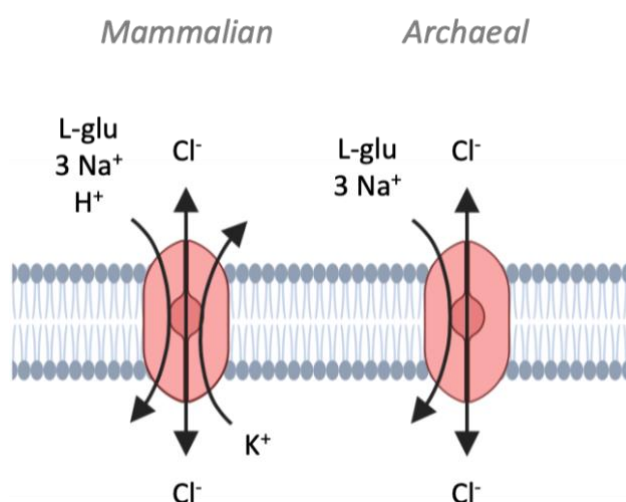


Figure 1.11 Comparison of the transport stoichiometry in mammalian EAATs (left) with archaeal glutamate transporters, GltTk and GltPh (right). Arrows show movement of substrate and ion transport.

1.5.1 General structure

The homotrimeric structure of the glutamate transporters was suggested even before the first crystal structure was solved for outward-facing Glt_{ph}. This revealed a

bowl-shaped structure with a large solvent-filled extracellular basin reaching halfway across the membrane. The three identical protomers formed a tip pointing to the intracellular cytoplasmic environment.¹¹¹ Each protomer consists of 8 transmembrane segments (TMs) and two helical hairpins (HP1 and HP2). These are divided into a scaffold (or trimerization) domain and a transport domain. The scaffold domain is composed of TMs 1, 2, 4 and 5 forming a propeller like shape to anchor the protein into the membrane. The transport domain is located between the propellers and is composed of TMs 3, 6, 7, 8, HP1 and HP2 which contain the substrate and ion binding sites (Figure 1.12).^{109,111,116}

It was initially suggested HP1 acts as a gate to the intracellular environment, and HP2 for the extracellular environment. However, several studies have since found HP2 acts as a gate to both with HP1 being involved in substrate binding.^{111,120–122} The structure of the archaeal homologues is very similar to the mammalian transporters, with the only differences being insertions and deletions where TM1 is split into two and TM8 into three helices in the EAATs and there is a long loop between TM4b and TM4c only seen in the mammalian transporters (Figure 1.12).^{83,86}

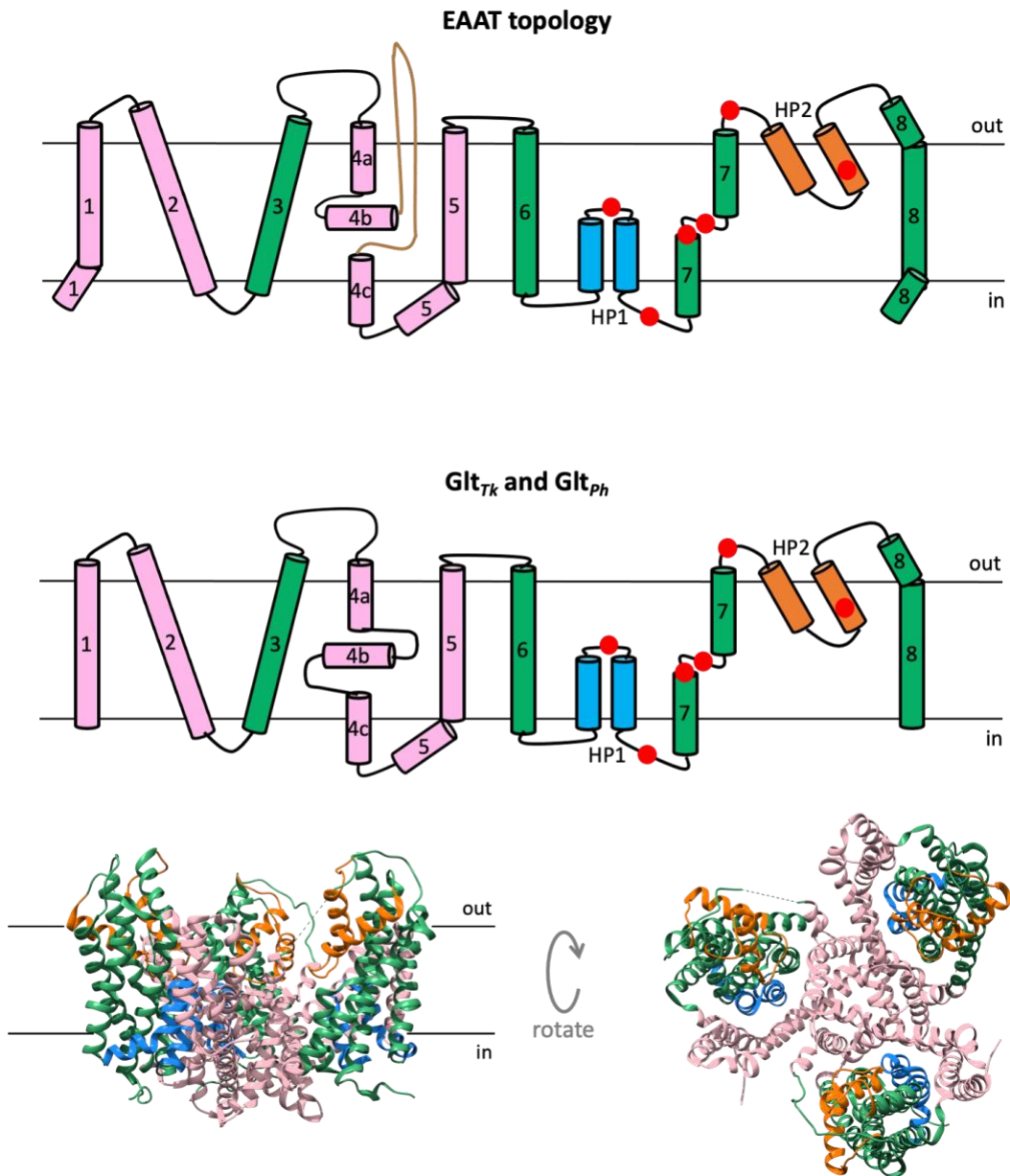


Figure 1.12 The mammalian EAAT and archaeal glutamate transporters, Glt_{Tk} and Glt_{Ph}, membrane topology. The scaffold domain is shown in pink and transport domain in green, HP1 in blue and HP2 in orange. The EAATs also have a loop between TM4b and TM4c shown in brown which isn't present in the archaeal homologues. Bottom: The 3D structure of Glt_{Tk} is shown in the membrane plane (left) and view from the extracellular side of the membrane (right), highlighting the homotrimeric structure (PDB 4KY0).

1.5.2 Binding sites

As mentioned previously, the binding sites in particular are highly conserved between both the archaeal and mammalian glutamate transporters. Between Glt_{Tk} and Glt_{Ph} the residues involved in substrate and Na⁺ binding are identical, appearing at slightly

different residue numbers, with those associated with Glt_{ph} shown in brackets. The substrate binding site for L-aspartate was identified as a polar chamber created by the tips of HP1, HP2, and by the sequence NMDGT motif on TM7 and the polar residues of TM8 forming a highly conserved motif (Figure 1.13). The amino group of aspartate is coordinated by R278 (R276) HP1, V358 (V355) HP2, D397 (D394) TM8 and T402 (T398) TM8; the carboxyl group of aspartate is coordinated by S280 (S278) HP1, N405 (N401) TM8; and β -carboxylate with T317 (T314) TM7, G362 (G359) HP2, and R401 (R397) TM8.^{111,116} The similarity between the substrate binding sites of Glt_{Tk}, Glt_{ph} and the EAATs make the differences in substrate preferences difficult to understand, particularly for the two archaeal homologues where there is no data for Glt_{Tk} with L-glutamate.

Within each protomer there are three Na⁺ binding sites, one for each ion required for the transport of glutamate across the membrane. The first reported crystal structures of Glt_{ph} revealed the location of two of the three sites,¹¹¹ with the third being identified in the a substrate-bound Glt_{Tk} crystal structure (Figure 1.13).¹¹⁶ The initial identification of Na1 and Na2 sites in Glt_{ph} was revealed by the co-crystallisation with thallium ions (Tl⁺), and confirmed to be the Na⁺ binding site by competition experiments with more recently reported structures corroborating these results. The Na1 site is buried deep under the aspartate binding site where Na⁺ is coordinated by the β -carboxylate of D409 (D405) TM8, main chain carbonyls of G309 and N313 (G306 and N310) TM7) and N405 (N401) TM8. Just under HP2, the Na2 site coordinates Na⁺ with the main chain carbonyls of S352, I353 and T355 (S349, I350 and T353) HP2 and T311 (T308) TM7.

The third Glt_{Tk} Na⁺ binding site was identified between TM7 and TM3 involving coordination of Na⁺ with the carboxamide of N313 and side chain carbonyl of D315 (N310 and D312) TM7, the hydroxyl groups of T94 and S95 (T92 and S93) TM3 and the main chain carbonyl of Y91 (Y89) TM3.¹¹⁶ This is consistent with the predictions made using molecular dynamic simulations of Glt_{ph}.¹²³ The Na1 and Na3 binding sites are in very close proximity (8 Å) shielded by N313, and M314 sits at the centre of the triangle formed between Na1, Na3 and the aspartate binding site with HP2. Given

the close proximity of the sites, it is unsurprising that cooperativity is observed in binding substrate and Na⁺.

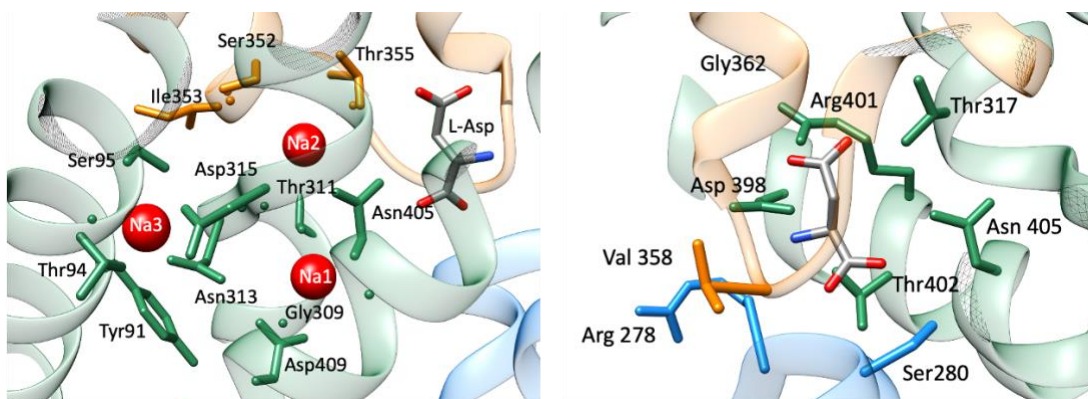


Figure 1.13 Cartoon representation of the sodium ion and substrate binding sites of Glt_{Tk} shown left and right respectively. Residues involved in ion and L-aspartate coordination are shown as sticks. Green represents transport domain, blue is HP1 and orange HP2 (PDB 5E9S).

1.5.3 Structural studies and biophysical characterisation of Glt_{Tk} and Glt_{Ph}

The first crystal structure of Glt_{Ph} was an outward-facing occluded conformation where the substrate binding site was closed to the intracellular and extracellular environments. This raised the question of how substrate molecules reached the binding site from both sides of the membrane and how access is controlled. A comparison made between substrate-bound, and inhibitor bound (DL-*threo*-β-Benzyloxyaspartic, DL-TBOA) Glt_{Ph} found very similar structures with the main difference being the positioning of HP2. In TBOA-bound Glt_{Ph}, HP2 was in an open conformation which exposed the substrate binding site to the extracellular environment. HP2 shifted 10 Å closer to the loop between TM3 and TM4 than when aspartate-bound, with the loop itself also shifting closer to HP2.¹¹¹ DL-TBOA is a competitive inhibitor with the same backbone structure as L-aspartate with an additional benzyl ring which cannot fit into the binding site (Figure 1.14). Therefore, binding of DL-TBOA traps Glt_{Ph} in an open conformation leading to the identification of HP2 as the extracellular gate. Steric clashes prevent the gate from closing due to lodging of the bulky benzyl group against the tip of HP2. This inhibits the necessary conformational changes to transport substrate molecules across the membrane such

that binding inactivates both archaeal and mammalian glutamate transporters. The gate must first be closed to transition between outward and inward facing states.

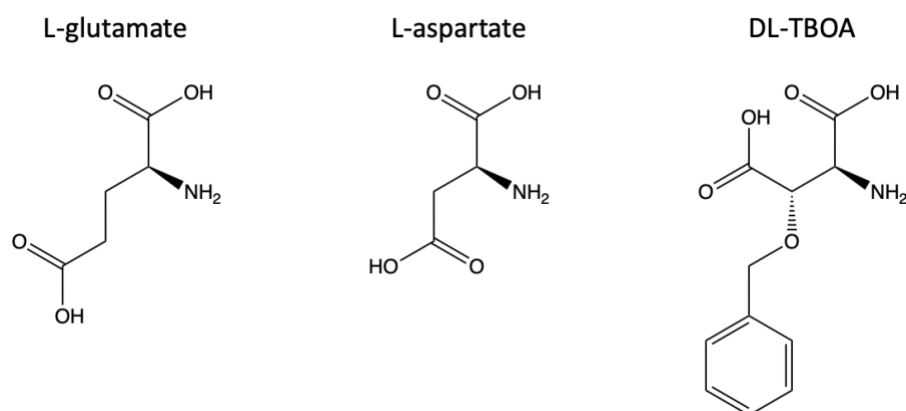


Figure 1.14 Structures of L-glutamate, L-aspartate and DL-threo- β -Benzyloxyaspartic (DL-TBOA)

Binding of TBOA was found to be coupled to two Na⁺ ions: Na1 and Na3 were in coordinating positions with the site of Na2 distorted due to HP2 being open.^{124,125} A Na⁺ bound crystal structure of Glt_{ph}, without aspartate present, showed HP2 in a less-open conformation compared to in the TBOA-bound crystal structures.¹²⁶ This is thought to allow access of aspartate to the substrate binding site, preventing conformational changes until this has occurred and the gate has shut.

An Inward-facing crystal structure of substrate-bound Glt_{ph} provided an insight into the transition between inward and outward facing states. To achieve this two residues (K55/A364) were cross-linked with Hg²⁺ to reveal movement of the transport domain 15 Å across the lipid bilayer to reach the inward-occluded state.¹⁰² This large scale movement of the transport domain had not been observed previously. The scaffold domain remained unchanged compared to outward-facing conformations as expected with an alternating access elevator mechanism. These results are corroborated by the published inward-open conformations of Glt_{Tk} in Na⁺ and inhibitor-bound states (Figure 1.15).¹²²

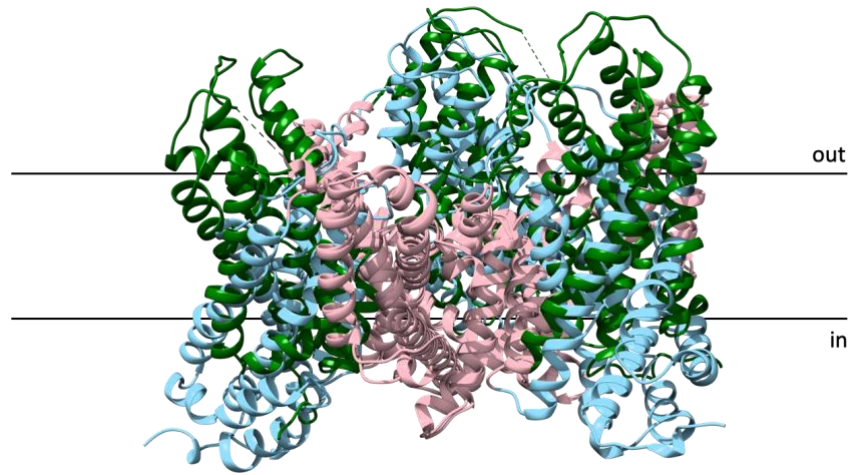


Figure 1.15 Superposition of homotrimeric Glt_{Tk} in an inward and outward facing conformation. In both structures the scaffold domain (pink) remains fixed, with the transport domain moving relative to allow access either side of the membrane. The transport domain is shown in an outward facing conformation (green, PDB 4KY0) and the second structure has two of the three protomers as inward open conformations with the third protomer outward occluded at the back (blue, PDB 6XWR).

To study Glt_{ph} using X-ray crystallography it was necessary to use either a single point mutant (R397C) or the double cysteine mutant K55C/A364C to form cross-links to constrain inward-facing conformations. In addition to the presence of all three Na⁺ in their binding sites, the crystallisation of Glt_{Tk} utilised wild-type (WT) protein which eliminated the reduced substrate affinity experienced for the Glt_{ph} mutants. However, structures of WT Glt_{ph} have been solved since. Glt_{Tk} also had higher expression levels than Glt_{ph}.^{108,109} This is likely to contribute to the higher resolution structures of Glt_{Tk} in the protein data bank.

The crystallisation of apo Glt_{Tk} in an outward-facing conformation provided an insight into substrate-free glutamate transporters, because all prior Glt_{ph} were substrate and/or Na⁺ bound (Figure 1.15).¹⁰⁹ Apo Glt_{ph} has been solved since, with both protein structures revealing distortion of all three Na⁺ binding sites.^{109,126} This suggests small-scale rearrangements occur to occupy all binding sites before transport across the membrane. In an inward-occluded substrate bound structure the side chain of M314 (M311 in Glt_{ph}) is exposed to the lipid bilayer, pointing towards the Na3 binding

site in apo protein. When substrate bound, M314 is involved in substrate binding where it points towards Na2 within interacting distance. M314 is part of the functionally important and highly-conserved NMDGT motif which couples induced conformational changes upon Na⁺ binding to accommodate binding substrate.^{109,111,116,126} Apo Glt_{Ph} and Glt_{Tk} have been crystallised in outward-facing, intermediate outward-facing and inward facing conformations where similar conformations of the transport domain were observed, with HP2 closed. This confirmed the movement of the transport domain as a rigid body relative to the scaffold domain. This provided an insight into the mechanism by which the transporters return back to an outward facing conformation for the next transport cycle after substrate and Na⁺ release.^{109,126}

The first structure of apo Glt_{Tk} also revealed a possible K⁺ binding site for the EAATs. N401 is a highly conserved residue among the glutamate transporters and has been proven to be essential for transport. This residue undergoes rearrangement between different binding states and with its surrounding residues was shown to create a pocket, proposed to be the K⁺ binding site, when the transport domain moves along the scaffold domain.¹⁰⁹ This is supported by mutation of the equivalent N401 residue in EAAT3 which resulted in no binding or transport of both aspartate and glutamate, and is thought to be essential for reorientation of the empty carrier.¹²⁷

After the initial apo outward-facing structure, Glt_{Tk} crystal structures were solved in the presence and absence of aspartate where the transport domain was in outward-occluded conformations for both.¹¹⁶ This enabled the location of the third Na⁺ binding site (as discussed in Section 1.5.2), in addition to well defined electron density for all loops and N-termini which were disordered in previously published Glt_{Ph} crystal structures. It was found the loop between TM3 and TM4 restricts movement of HP2, only allowing small scale movements needed to control access to the binding sites. A comparison of D- and L-aspartate bound Glt_{Tk} was used to investigate the difference in orientation and coordination of the enantiomeric substrates, which have similar affinities (as determined by ITC).¹¹³ D-aspartate only required small rearrangements in the structure of the binding sites in the

outward-occluded state. Given the residues involved in substrate binding are highly conserved, the identical hydrogen bonding networks formed were unsurprising. The only subtle changes required to accommodate the other isomer were in the position of the C α and β -carboxyl group of the substrate, with the same Na⁺ binding sites observed.¹¹³ This was the first crystal structure of an amino acid transporter with enantiomeric substances characterised to show absolute orientations. The K_D value of D-aspartate was 4–6 times higher than L-aspartate, which could be a result of the higher dissociation rate deduced from kinetic studies of Glt_{ph}.^{113,128}

Studies of the cross-linked Glt_{ph} variant (K55C/A364C) in nanodiscs were used to investigate the gating mechanisms on the intracellular side of the membrane by crystallising inward facing Glt_{ph} in different binding conditions (apo, Na⁺, substrate bound and inhibitor bound).¹²¹ These structures resolved the N-terminus which anchored into the lipid membrane, this detail was previously unseen in other structural studies. Overall, there was agreement between Glt_{ph} structures in the nanodisc environment and those previously published of the protein in detergent micelles. Inward facing substrate bound Glt_{ph} had a closed gate (HP2) in the same orientation as previously observed for apo inward-facing Glt_{ph}.¹²⁶ The main difference between the inward-facing structures was the degree by which the transport domain swings out away from HP2 and the scaffold domain.¹²¹ This movement is proposed to allow access to the substrate binding site as previously seen for other inward-facing Glt_{ph} structures.¹²⁹ However this raised the question of whether the transport domain swings out first to provide space for HP2 opening; or is the swinging movement alone sufficient for binding site access on the intracellular side.

In this study two inhibitors were used: TBOA and TFB-TBOA ((2S,3S)-3-[3-[4-(trifluoromethyl)benzoylamino]benzyloxy]aspartate) where the difference is the presence of an extra benzyl ring on the latter. Inward facing Glt_{ph} bound to TBOA showed a mostly closed HP2 gate, with a small opening between the tips of HP1 and HP2.¹²¹ The separation between the N-terminal arm and C-terminal arm altered one of the Na⁺ binding sites, giving further evidence to the different Na⁺ binding stoichiometry previously proposed. Interestingly, DL-TBOA bound Glt_{ph} in an outward

facing confirmation exhibited an open gate. However, when bound to TFB-TBOA in an inward-facing conformation, Glt_{ph} adopted the same conformation of outward facing TBOA-bound Glt_{ph}, including the open HP2 gate.^{111,121} This confirmed HP2 acts as an intracellular gate as well as an extracellular gate.

The asymmetric conformations of the trimeric glutamate transporters was highlighted in a recent study of Glt_{Tk} in nanodiscs where four of the five structures were composed of two different protein conformations (Figure 1.16).¹²² These previously undetected conformations of individual protomers help give insight into the transport mechanism. The only symmetrical arrangement was observed for Glt_{Tk} in the presence of excess substrate where a single intermediate outward occluded conformation was observed, with HP2 closed. The conformations of the Na⁺ binding site residues differ significantly between apo and substrate bound structures. Every inward open conformation in this study was assigned to be Na⁺ bound which is proposed to prevent substrate leakage. Each protomer in the presence of substrate, inhibitor (DL-TBOA) and apo favoured outward-facing conformations. This asymmetry has been supported by biochemical and biophysical studies too.^{103–107} For the Na⁺ bound structure, one of the protomers was identical to previously published apo Glt_{Tk} where the Na⁺ binding sites are deformed and HP2 closed. The other two protomers in inward-open conformations had an open gate (HP2) with the substrate binding site accessible, and density corresponding to Na⁺ in Na1 and Na3. Na2 was not properly formed due to HP2 being open. The formation of the high-affinity aspartate binding site upon partial Na⁺ binding has been seen previously.^{109,116,124}

Two structures were found under sub-saturation conditions, both of which included inward-facing and intermediate outward occluded conformations in ratios of 1:2 and 2:1 respectively. This means under unsaturated conditions there could be more states possible that have not previously been observed. One interesting observation was the outward-open conformation only appeared in the inhibitor bound structure, and for two of the three protomers. The third protomer was Na⁺ bound in an inward facing state despite the presence of excess DL-TBOA.

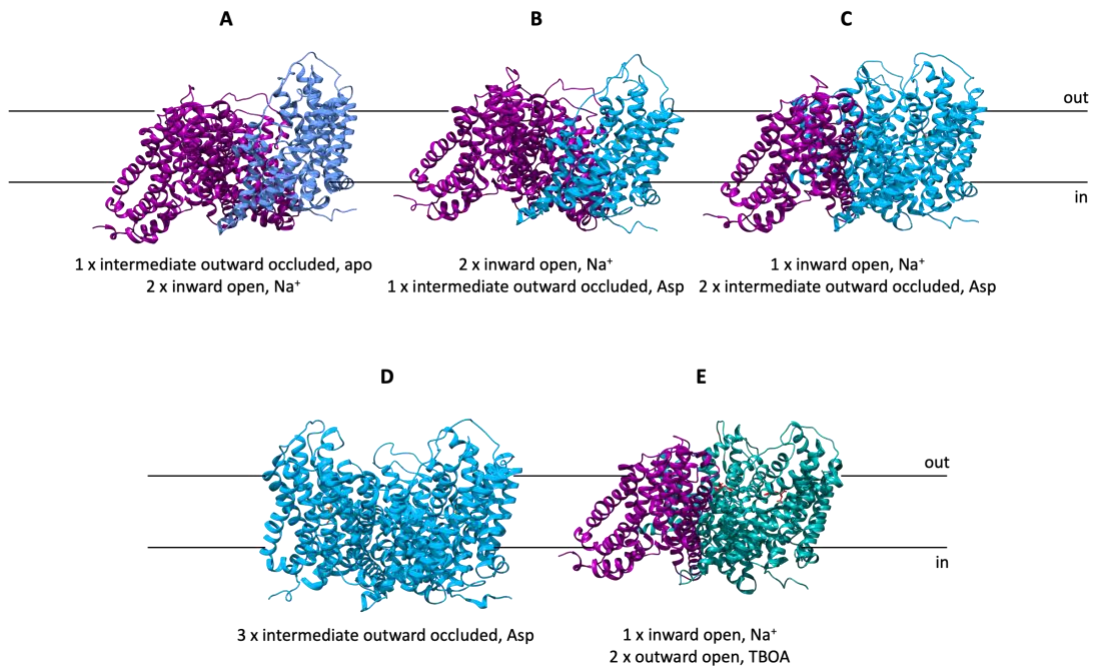


Figure 1.16 Glt_{TK} cryo-EM structures in different conformational states highlighting the asymmetry of the homotrimeric protein. A) Na⁺ bound, B) Unsaturated aspartate-bound (2 in: 1 out), C) Unsaturated aspartate-bound (1 in: 2 out), D) Saturated aspartate bound, E) inhibitor (TBOA) bound. Intermediate outward occluded protomers are shown in blue (cornblue for apo, sky blue for substrate bound and dark cyan for inhibitor bound) and inward open Na⁺ bound protomers in purple. PDB codes: 6XWR, 6XWO, 6XWP, 6XWQ and 6XWN (A to E).

In both mammalian and archaeal glutamate transporters, substrate transport is accompanied by a thermodynamically uncoupled chloride conductance. A recent crystal structure of Glt_{ph} used three different cross-linked residues chosen for their proximity in the outward facing, intermediate outward facing and inward facing structures. Within each cross-linked pair, one residue was located on the transport domain and the other on the scaffold domain¹¹⁷ These structures revealed an open channel state where an aqueous cavity formed during the transport cycle. The identification of two hydrophobic regions which are highly conserved in all glutamate transporters at the extracellular and intracellular regions were proposed to act as gates, helping to map out the complete transport cycle to include a chloride conducting state. Previous electrophysical studies have identified residues that line the proposed channel at the interface between the transport and scaffold domains.^{117,118,126,130} A vertical twisting movement of the transport domain as the protein transitions from an outward to an inward facing structure creates the

observed chloride channel. A recent study of Glt_{7k} used solid-supported membrane (SSM) electrophysiology, ITC and transport assays to confirm the presence of the gated anion channel and found conductance to increase in Glt_{7k} S280R whilst the L-aspartate transport rate decreased.¹¹⁹ Residue 280 is part of a highly conserved triple serine motif which is involved in substrate binding and dispatch of sodium ions to their binding sites.^{131,132} The protein architecture remained the same so the increased conductance could be a result of widening of the anionic channel.

The Na⁺ dependency of L-aspartate transport for the glutamate transporters is well established, with several studies confirming substrate transport does not occur in the absence of Na⁺. Boudker et al. published results of thermodynamic studies alongside the first crystal structure of Glt_{ph}.¹¹¹ These revealed K_D values in the nanomolar range for L-aspartate, with a Hill co-efficient close to one. The latter therefore indicated each protomer underwent binding events independently of each other. Several studies since have supported both the K_D determinations, and the lack of cooperativity between protomers including the use of fluorescence measurements.¹²⁸ However, a recent study using saturation transfer nuclear magnetic resonance (STD-NMR) spectroscopy found evidence of negative cooperativity.¹³³ This means binding of substrate to one protomer makes binding to another less likely, a phenomenon which was only observed in liposomes and not detergent micelles.

Small molecule fluorescence energy transfer (smFRET) studies the energy transfer between two fluorophores to measure distances between 1 and 10 nm. This has been applied to Glt_{ph} reconstituted into proteoliposomes with the aim to gain information about the transport mechanism and associated conformational dynamics. In the first smFRET study of Glt_{ph} Cy3 was used as a donor fluorophore with Cy5 as the acceptor – where Cy3 and Cy5 are FRET cyanine dyes. Cysteine variants were used to attach the fluorophores.¹⁰⁵ Fluorescence microscopy confirmed the scaffold domain remained fixed during the transport cycle, with the transport domain mobile and preferentially in an outward-open conformation, as observed using other methods.^{134,135} The transport domain was seen to fluctuate between periods of dormancy and rapid

transitions. During the dynamic periods, the transport domain sampled multiple conformations for apo Glt_{ph}. Upon substrate binding the lifetime of the inward and outward facing states was increased and the interchange between conformations was less frequent than observed for apo. The frequency of rapid dynamics decreased further, by more than 80%, on addition of inhibitor (DL-TBOA) showing the dynamics are a result of substrate translocation across the membrane.¹⁰⁵ This provided evidence of allosteric coupling between the substrate binding site and domain interface. Faster translocation and slow isomerism led to the conclusion that the separation of the transport domain from the scaffold domain was the rate limiting step. This separation is referred to as the unlocking of the transport and scaffold domain needed for conformational changes. Protomers were found to move independently to each other with heterogeneous conformational fluctuations with structurally different short-lived states to those observed in crystal structures.

A second smFRET study used 'humanised' variants R276S/M395R where faster dynamics and transport rates were observed.¹²⁹ The variant showed a 1,000 fold decrease in affinity and a 4 fold faster uptake rate for L-aspartate compared to WT Glt_{ph}. This could be explained by the enlarged binding pocket in the inward-open state of the humanised variant, which was supported by x-ray crystallography structures and molecular dynamic simulations. This study also confirmed the rate limiting step as the unlocking of the domains for WT, with long dormant and occasional dynamic periods. In the humanised variant the rate limiting step became the transport domain movements due to the increased stability of the outward facing state when substrate bound. R276S/M395R are not part of the substrate binding site, but cause changes in both dynamics and substrate affinity which further adds to the evidence of coupling between substrate binding and protein dynamics.

The use of triply labelled species (either one donor, two acceptor or vice versa) further confirmed the independent conformational changes and unsynchronised dynamics of Glt_{ph}. In the absence of aspartate, the transport domain cycled between outward and intermediate, or inward and intermediate states, preventing complete movement of the transport domain across the membrane and therefore preventing

Na⁺ leakage.¹⁰³ An L-aspartate/glutamate-binding periplasmic protein was used to develop a FRET-based sensor to monitor the uptake of L-aspartate by Glt_{ph} in liposomes. Individual transporters were found to function at different rates, ranging from a few to hundreds of seconds per cycle. A molecular memory was found where transporters preserved these rates over successive uptake cycles.¹³⁶ A further smFRET study showed a kinetic heterogeneity between single transporter activity and elevator dynamics. The transport cycle was shown to have two kinetically heterogeneous phases; substrate translocation from the outward- to inward-facing states and substrate release from the inward facing state, both of which can be rate limiting. The former has also been proposed as a rate limiting step in previous studies.^{104,105} Mutations in HP2 affected both steps reinforcing the critical role in the transport cycle. However, homologous glutamate transporters have different rate limiting steps so more research needs to be done in this area. For the EAATs, the rate limiting step is proposed to be the return of the K⁺-bound transport domain to the outward facing state.^{137,138}

The high-energy transition state of Glt_{ph} was shown to be rate limiting by FRET studies using gain-of-function mutations in HP2.¹³⁹ These states are crucial to understand kinetic mechanism and are often not solved using structural methods due to their low population. Using linear free energy relationship (LFER), the transition state structure was characterised during translocation. The gain-of-function mutants were identified by comparing homologues from different bacteria and using functional assays and smFRET those with faster uptake rates and decreased affinity were identified (in addition to the 'humanised' variant in other studies).^{129,139} HP2 mutants had the same populations of inward and outward facing states in substrate bound conditions, with similar affinities and gating roles in the two states. LFER showed the transition state in an inward facing state, so multiple attempts may be needed to cross the high-energy barrier to get there from the outward facing state. HP2 mutations affected both forward and reverse rates which supported the one-gated model of substrate transport, with protomers acting independently.

High speed atomic force microscopy (HS-AFM) was used to study the elevator mechanism of Glt_{ph} in liposomes, confirming at least two Na⁺ bind before L-aspartate.¹⁰⁴ As suggested by previous studies, the elevator dynamics only occur in apo or substrate bound states. In the presence of Na⁺ only, the dynamics are stalled with significant reduction in the number of moving protomers to prevent leakage. Each protomer was shown to operate individually, with inward facing conformations short-lived and high-energy. These results correlated with the dynamic motions and dormancy suggested by smFRET studies discussed previously, with no cooperativity observed between protomers.^{104,105,129}

There are significantly fewer biophysical studies of Glt_{Tk} compared to Glt_{ph}. Often cryo-EM and crystal structures are published with simulations or information on kinetics. For example, in the report of the first substrate-bound crystal structure, ITC was used to confirm three Na⁺ bind per aspartate like Glt_{ph}, in addition to molecular dynamic simulations and binding assays to confirm coupling between Na⁺ and L-aspartate. Structural studies provide a qualitative view of the transport mechanism and explanation of binding order, but quantitative studies are needed to reveal the full kinetic mechanism.

Molecular dynamic simulations and transport equilibrium potentials were used to inform on the role of a conserved methionine (M314 in Glt_{Tk}) in the Na⁺ coupling mechanism.¹⁴⁰ This residue had previously been shown to be key for substrate turnover rate and specificity and is involved in Na⁺ coordination. Mutations have been shown to diminish binding cooperativity between Na⁺ and substrate but the influence on thermodynamics was unclear. This study found methionine to foster specific kinetics in the binding reaction which influences the turnover rate, in addition cooperativity of binding isn't essential for stoichiometry enforcement. When M314 was mutated, the stoichiometry remained the same, but the binding cooperativity was impaired. This informed on the mechanism of substrate transport, where only selected occupancy states allow occluded conformations.

Studies of the transport kinetics of Glt_{Tk} in liposomes found a six-fold difference in turnover rate compared to Glt_{Ph} with values of 0.9 s⁻¹ and 0.14 s⁻¹ respectively.¹⁴¹ Synthetic nanobodies were used to inactivate populations in a right-side out conformation in the liposome, so only inside-out orientated protein was studied. This was similar to the cross-linking experiment on Glt_{Ph} discussed previously to measure transport kinetics, with consistent results observed.¹⁰² The differences observed are proposed to be a result of the small structural differences away from the binding site, because all steps after binding the last Na⁺ contribute.

1.5.4 Previous studies of Glt_{Ph} using EPR spectroscopy

To date there are no published electron paramagnetic resonance (EPR) spectroscopy studies of Glt_{Tk}, with only a handful of studies published for closely related Glt_{Ph}. The first EPR study of Glt_{Ph} used double cysteine mutants (V355C-S279C; V355C-P129C; and A353-L130C) in liposomes to study the conformational changes in HP2 using both continuous wave (cw) EPR and pulsed EPR.¹⁴² Previous crystal structures solved in DL-TBOA and L-aspartate bound states showed changes in the position of HP2 but the apo and Na⁺ structures had not been solved.^{102,108,111} Cw-EPR spectroscopy was used to study the variants both maximally-labelled and minimally-labelled where the latter was approximately one spin label per pair of residues. Distance changes between residues were also monitored using pulsed EPR spectroscopy to follow conformational changes during the transport cycle.¹⁴² In an apo state, HP2 resembled that of substrate bound Glt_{Ph}. Binding of Na⁺ initiated HP2 opening, which stabilised the structure and HP2 then closed when L-aspartate was bound. The closure of HP2 enabled the movement of the empty binding pocket to complete the transport cycle. These results differed to previous suggestions where HP2 was proposed to be open in an apo state.¹⁰² Upon binding DL-TBOA, like with Na⁺, HP2 was open, agreeing with the crystal structures. Overall, this study exhibited cation specific conformational changes with general agreement to crystal structures, proposing a binding mechanism to summarise these results. This also showed no substrate transport can occur in the absence of Na⁺ due to the closed HP2 in the apo state prohibiting substrate entry into the binding site, as previously suggested in other studies.^{111,138,143}

Two further EPR studies were published in 2013 with the focus on elucidating large-scale conformational changes of Glt_{ph} in both detergent micelles and proteoliposomes.^{134,135} Both studies included a variety of cysteine variants, spin labelled with MTSL, located on both the scaffold and transport domains. At the time the crystal structures available for Glt_{ph} were symmetric either outward or inward facing states. DEER experiments confirmed that the transition between inward and outward facing states involves large-scale movements of the transport domain, with the scaffold domain forming the stable core of the protein. Multiple conformations were captured in different binding states (apo, Na⁺, substrate bound) with HP2 local movements linked to substrate loading and expulsion. No cooperativity of Na⁺ and substrate binding between each of the protomers was detected. Georgieva et al. found a 10-20% increase in outward-facing populations in liposomes, with some variants showing single dominant conformations as a result of interactions between the interface of the two domains.¹³⁴ Hänelt et al. found a preference for intermediate conformations for the liposome environment, compared to inward and outward facing in detergent micelles.¹³⁵ The main discrepancy between the studies is the preferred conformation in a specific state, which is likely to depend on where the spin-labelled residues are located. The equilibrium constants determined for outward and inward facing states were a similar magnitude which was consistent with the multiple occupied conformation states. Shorter distance distributions were observed in a lipid environment as a result of a more compact structure, but further research is needed into how factors such as lipid composition and thickness of the bilayer affect the Interactions.

An additional EPR study was used to investigate the cooperativity in substrate binding of heteromeric Glt_{ph} variants. *In vitro* multimeric assembly of Glt_{ph} required a mixture of WT and double cysteine variant (V216C–I294C) subunits produce a mixture of homomeric and heteromeric proteins.¹⁴⁴ WT Glt_{ph} does not contain native cysteine residues and therefore cannot bind MTSL, resulting in deconvolution of complex spectroscopic data for the heteromeric protein. DEER measurements of homomeric Glt_{ph} V216C–I294C were compared to heteromeric protein to reveal more defined distance distributions for the latter. This is a result of fewer distances measured due

to the reduction in the number of spin labelled sites. The heterotrimer also adopted an inward facing conformation when apo and substrate bound, contrasting to the more typical outward facing structure observed under these conditions.

The most recent EPR study of Glt_{ph} elucidated the specific roles of Na1 and Na3 in substrate binding and translocation. Continuous wave EPR spectroscopy was used in combination with fluorescence assays to monitor HP2 movement in different binding states.¹⁴⁵ EPR results confirmed Na⁺ binding to Na1 partially opens HP2, and the Na3 site must be occupied for the conformational switch in the NMDGT motif to occur. Therefore, both Na1 and Na3 must contain Na⁺ for high-affinity L-aspartate binding. This work used cysteine residues to attach MTSL to otherwise 'WT' protein. An additional variant was created to perturb Na⁺ coupling of HP2 gate opening – D405N which is the only side chain residue involved in the Na1 binding site. Continuous wave EPR studies indicated HP2 opening could occur in the absence of Na⁺ which allowed L-aspartate to bind to apo Glt_{ph}. This mutation was proposed to partially mimic Na⁺ binding at Na1, therefore the binding of L-aspartate to apo D405N is equivalent to binding to the Na1 occupied transporter. This led to the proposal of an alternative low affinity transport mechanism in the presence of low L-aspartate levels and high Na⁺ concentrations as discussed in Section 1.5.5.

1.5.5 The proposed transport mechanism

Various studies of Glt_{Tk} and Glt_{ph} have led to elucidation of the proposed elevator alternating access mechanism. Techniques employed include crystal and cryo-EM structures, molecular dynamic simulations, EPR spectroscopy, high-speed atomic force microscopy (AFM) and single molecule FRET.^{103–105,116,134,135} This model involves the transition between outward facing and inward facing conformations via occluded intermediate states. The high heterogeneity and short lifetime of intermediate states make them hard to crystallise, with only intermediate outward occluded states of the glutamate transporters solved. The preference for different conformational states varies between studies and model systems. Huang et al. found less Glt_{ph} protomers in nanodiscs sampled an intermediate outward facing state compared to an outward

open conformation; contrasting to Glt_{TK} (also in nanodiscs) where intermediate outward facing conformation was preferred over outward open under the same binding conditions.^{122,146} However, this could be a result of the labelled M385C Glt_{Ph} mutant studied compared to WT Glt_{TK}. This highlights the importance of labelling at different positions to get an overall consensus on preferred conformations in the different binding states.

It is proposed that in an apo state the extracellular gate (HP2) of the glutamate transporters is closed in an outward-occluded conformation. Two Na⁺ ions bind to Na1 and Na3 which causes HP2 to open, in addition to a local rearrangement to form the substrate binding site. Despite the tighter binding observed for Na3, Na1 binds first causing slight opening of HP2 for subsequent binding to Na3. Na3 was found to have the shortest interaction distances between the protein and cation, and Na⁺ binds tighter based on free energy calculations.¹¹⁶ Occupation of the Na3 site stimulates a conformational switch of the NMDGT motif. Aspartate binds to the outward-open conformation, causing a transition into outward-occluded state with HP2 closed to prevent substrate leakage. The final Na⁺ binds to Na2 to secure the extracellular gate shut before substrate translocation occurs. Substrate transport is cooperative in Na⁺ binding, using the electrochemical Na⁺ gradient for an elevator type movement from an outward facing to inward facing state.^{116,124,141} The transport domain moves as a rigid body across the scaffold domain with a 37° rotation and 16–18 Å translation, using the loops between TM2–3 and TM5–6 as hinges.¹⁰²

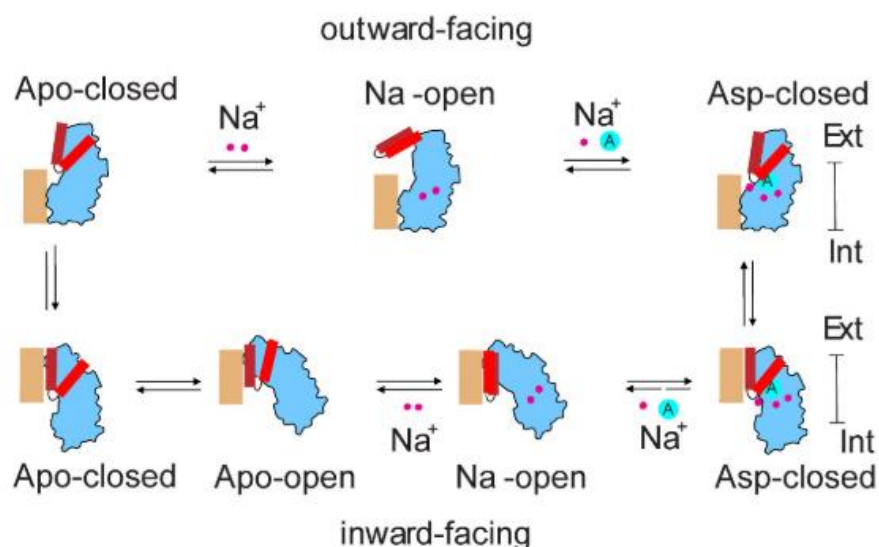


Figure 1.17 A proposed alternating access mechanism for Glt_{ph} highlighting the gating mechanism on both sides of the membrane. HP2 is shown in red, with the transport domain in blue and scaffold domain in wheat. In an apo-closed state (top left), binding of two sodium-ions causes the gate to open (Na-open). This enables the substrate to enter the binding site, followed by the third sodium-ion. The gate closes (Asp-closed) to preventing substrate leakage. The TMs making up the transport domain undergo rearrangements to form an inward facing conformation (Asp-closed, bottom right) where gate-opening is followed by release of substrate and the last sodium ion to bind. The final two sodium ions are also released to the intracellular environment. Gate-closure returns the protein to the apo-closed conformation where it transitions from inward to outward facing (bottom to top left) and the cycle repeats. Figure from Wang and Boudker 2020.¹²¹

The low affinity of Na₂ means Na⁺ is the last to bind and the first to be released, this is followed by the intracellular gate opening for substrate release.^{116,147} Both HP1 and HP2 have been proposed as the intracellular gate, with the most recent studies supporting a one-gate mechanism where HP2 acts as a gate on both sides of the membrane (Figure 1.17).^{111,120–122} After substrate release, Na₁ and Na₃ are emptied and a conformational switch of the NMDGT motif causes HP2 to close. The empty inward-occluded facing conformation resets to the outward facing state ready for the next transport cycle, which is proposed to occur in the same movement as substrate transport across the membrane.

A recent study of Glt_{Ph} proposed an alternative mechanism when binding events occur in a state of high Na^+ and low L-aspartate. In the alternative pathway Na^+ binds to Na1, followed by L-aspartate and then Na^+ at Na3 followed by Na2 (Figure 1.18).¹⁴⁵ Despite the proposed current mechanism, further experiments are needed to gain an in depth understanding. The order of Na1 and Na3 binding has been elucidated, however the pathway of the second Na^+ binding event is under debate. The two main suggestions are i) the first Na^+ bound to the Na1 site moves into the Na3 site, and a second Na^+ refills Na1 or ii) the second Na^+ binds to Na3 directly with the other Na^+ at Na1 remaining fixed.¹⁴⁸

Lipid moieties have been found in spaces between the transport and scaffold domain. Protein-lipid interactions are proposed to balance energetic penalties, however their specific contribution to the alternating access mechanism is unknown.^{121,122,129,149} The mechanism of chloride conductance by the glutamate transporters is ambiguous due to limited data.^{117,119} Although the presence of the chloride channel has been proven in the mammalian and archaeal transporters, there are currently no proposed mechanisms of chloride conductance.

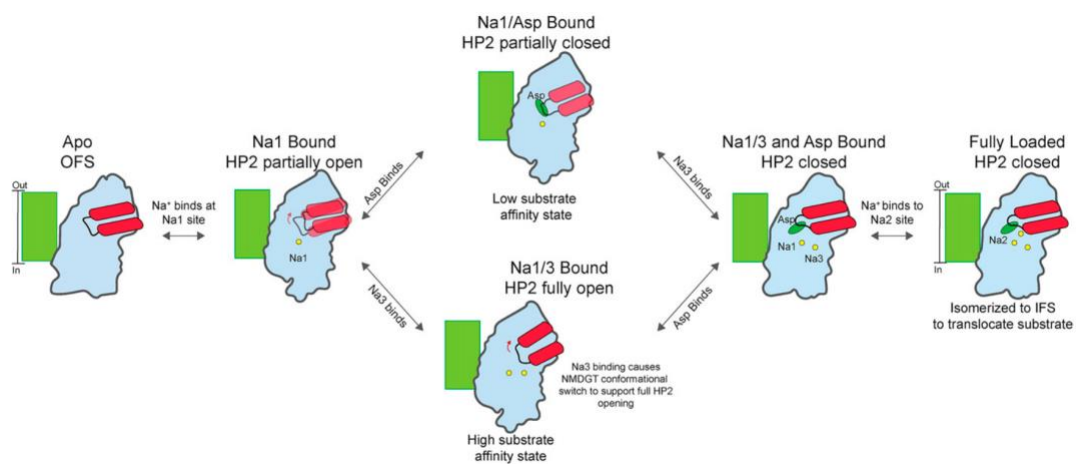


Figure 1.18 The coupled binding of sodium and aspartate by Glt_{Ph} during the alternating access mechanism via two proposed pathways: (top) during high sodium and low aspartate concentrations; (bottom) during low sodium and high aspartate concentrations. The binding events and gate (HP2, red) movements are detailed in the figure, with substrate in green and sodium ions in yellow. Figure from Riederer et al.¹⁴⁵

1.6 Aims and objectives

The aims of this thesis were to elucidate the mechanism and associated conformational changes of Glt_{Tk} and Glt_{Ph} using EPR spectroscopy. Multiple labelling sites were used to give an overall consensus of preferred conformations in the different binding states. Cysteine residues were introduced into different TMs to report on both the overall movement of the protein, but also in the localised region of the variants individually. This included studying Glt_{Tk} in different membrane mimetics to investigate the effect of the spin-labelled protein environment on the protein dynamics. This included detergent micelles, proteoliposomes, nanodisc and styrene maleic acid lipid particles (SMALPs). Saturation transfer difference (STD) NMR spectroscopy was used to investigate the dissociation constants, K_D , of Glt_{Tk} and Glt_{Ph} with L-aspartate and L-glutamate.

2 An introduction to Magnetic Resonance

Spectroscopy is the study of the absorption, emission and scattering of electromagnetic radiation. Spectroscopic techniques are often used to investigate the structural composition of a sample through detection or quantification of its components. When there is an absorption of electromagnetic radiation, a transition between two discrete energy states occurs. This relationship is described by Planck's equation (Equation 2.1), where ΔE is the difference in energy between the two states, h is Planck's constant ($6.62 \times 10^{-34} \text{ m}^2 \text{ kg s}^{-1}$) and ν is frequency of the applied radiation.

$$\Delta E = h\nu$$

2.1

Transitions occur from a lower to higher energy level when the atom or molecule absorbs radiation at the correct frequency, thereby inducing the transition (Figure 2.1). This is displayed as a spectrum where the absorption of radiation is typically given as a function of frequency or wavelength.

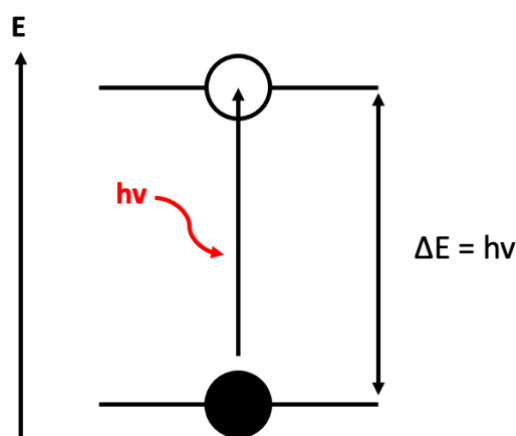


Figure 2.1 Absorption of electromagnetic radiation leads to a transition between two discrete energy states. The energy gap between the levels, ΔE , must equal the frequency of the electromagnetic radiation ($h\nu$) for the transition to occur.

Electromagnetic radiation contains both electric and magnetic field components. In most spectroscopic techniques, the electric field component of radiation interacts with electric dipole moments in the sample causing a transition. In contrast, magnetic

resonance techniques require permanent magnetic dipoles (from electrons or nuclei) to be present. Typically, an external magnetic field is applied to lift the degeneracy of the magnetic microstates and induce a population difference between them. Resonance occurs when the energy of the incident photon matches the energy splitting of any 2 of the microstates satisfying the selection rules for magnetic dipole transitions $\Delta m_{s/l} = \pm 1$ (where m_s and m_l are spin quantum numbers of electrons and nuclei respectively).¹⁵⁰ This thesis will focus on two magnetic resonance techniques: Nuclear Magnetic Resonance (NMR) spectroscopy and Electron Paramagnetic Resonance (EPR) spectroscopy.

The electron is a negatively charged particle which is characterised by spin - an intrinsic angular momentum. Spin angular momentum, S , is a vector of length $\sqrt{S(S+1)}$ with allowed projections onto an axis of $+S$ to $-S$ in integer steps giving $2S + 1$ projections in total. The allowed orientations are given by the electron spin angular momentum quantum number, m_s . The electron spin angular momentum always has an associated magnetic dipole moment, μ_s . This arises from spin and is given by Equation 2.2, where g_e is the free electron g-factor (2.0023, a dimensionless factor) and μ_B is Bohr magneton ($9.274 \times 10^{-24} \text{ J T}^{-1}$).^{150,151}

$$\mu_s = g_e \mu_B S \quad 2.2$$

Atomic nuclei are composed of protons and neutrons, which like the electron have mass and charge. Protons are positively charged, and neutrons are neutral. Nuclei have the intrinsic property of nuclear spin angular momentum, I , which is analogous to the angular momentum of an electron, S . The magnitude of I is given by $\hbar\sqrt{I(I+1)}$ where $\hbar$ is $h/2\pi$. Like an electron, there are allowed projections onto an axis of $+I$ to $-I$ in integer steps. Nuclei also generate a magnetic field as they precess, resulting in a magnetic moment μ_l proportional to I and nuclear gyromagnetic ratio, γ (Equation 2.3).

$$\mu_l = \gamma I \quad 2.3$$

2.1 Continuous wave EPR spectroscopy

2.1.1 EPR Spectrum acquisition

In most spectroscopic techniques, absorbance is measured as a function of frequency. However, in EPR spectroscopy the absorbance is very weak as a result of small population differences in the spin states. This means a strong magnetic field is required to observe the transitions. To record a continuous wave (cw) EPR spectrum a constant microwave frequency is applied, and the magnetic field is swept until the resonance condition is met and a signal is recorded. The resonance condition occurs when the gap between the energy levels is equal to the microwave radiation (discussed in Section 2.2.2).

To overcome the weak signals, the sample tube is placed into the cavity which is part of the resonator. Here the microwave field is used to achieve the resonance condition. Depending on the microwave frequency being used a different cavity will be required. Most of the cw-EPR spectra recorded in this thesis are at X-band (9.5 GHz) which is the equivalent to a wavelength of 3 cm. The cavity dimension used for an X-band resonator is 3 cm. The sample tube is positioned within the cavity to achieve maximum absorption of microwaves with minimum electrical energy, helping to amplify the weak signals. An iris screw is used to fine tune the number of microwaves entering the cavity and is used to critically couple the spectrometer. A klystron is used as the microwave radiation source, with waveguide channels to direct the radiation into the cavity.^{152,153} The resonator is sandwiched between two electromagnets where the field is varied depending on the current running through the coils. A continuous flowing water source keeps the electromagnetic coils from overheating. The signal is recorded as the amount of microwaves being reflected out of the resonator at a given magnetic field strength.¹⁵² This reflected power makes the signal easier to distinguish from the background, compared to if the absorbance of the sample was measured directly. The first derivative line shape comes from the field modulation which increases the signal to noise.

2.1.2 Site-directed spin labelling of proteins

Some biological systems already contain unpaired electrons, such as metal containing cofactors. However, most membrane transporter proteins are diamagnetic and as a result are EPR silent. Site-directed spin labelling (SDSL) is a structural biology tool used to attach a spin label to a specific position on a protein or biomolecule, allowing the system to be studied using EPR spectroscopy. This technique is independent of the size of the protein and can be applied in different protein environments.

The spin label acts as a reporter ligand for the molecule, revealing information such as local conformational dynamics and minor population states which may not be seen using techniques such as X-ray crystallography and cryo- electron microscopy (cryo-EM). There are a wide range of spin-labels available including transition metal ions, nitroxides and trityl radicals.^{154,155} The most commonly used spin label for membrane transporter complexes is the small nitroxide radical (1-Oxyl-2,2,5,5-tetramethyl- Δ^3 -pyrroline-3-methyl) Methanethiosulfonate (MTSL). The sensitivity of the spin-label to its environment is a result of anisotropy of the hyperfine interaction.¹⁵⁵ If two or more spin labels are present, the distance between them can be measured to get information about the structure and function of the system. The nitroxide part of MTSL contains N-O where the unpaired electron is present in a π orbital. The unpaired electron can be delocalised into the heterocyclic ring. MTSL contains a thiosulfonate group as a reactive linker which targets cysteine residues present in the protein to form a covalent, disulfide bond (Figure 2.2). However, thiosulfonate cannot be used under reducing conditions so alternative spin labels which contain maleimide or iodoacetate reactive linkers must be used instead¹⁵⁵.

Some proteins do not contain native cysteine residues, or they are located in unfavourable positions, so they must be selectively introduced (and removed if necessary) using site directed mutagenesis. Care must be taken to ensure the structure and functionality of the protein is not compromised by these mutations. In some cases, introducing cysteine residues may not be possible so alternative spin labelling methods must be used such as the introduction of unnatural amino acids¹⁵⁶.

This thesis uses MTSL spin labelling of selected cysteine residues so alternative labels and methods will not be discussed.

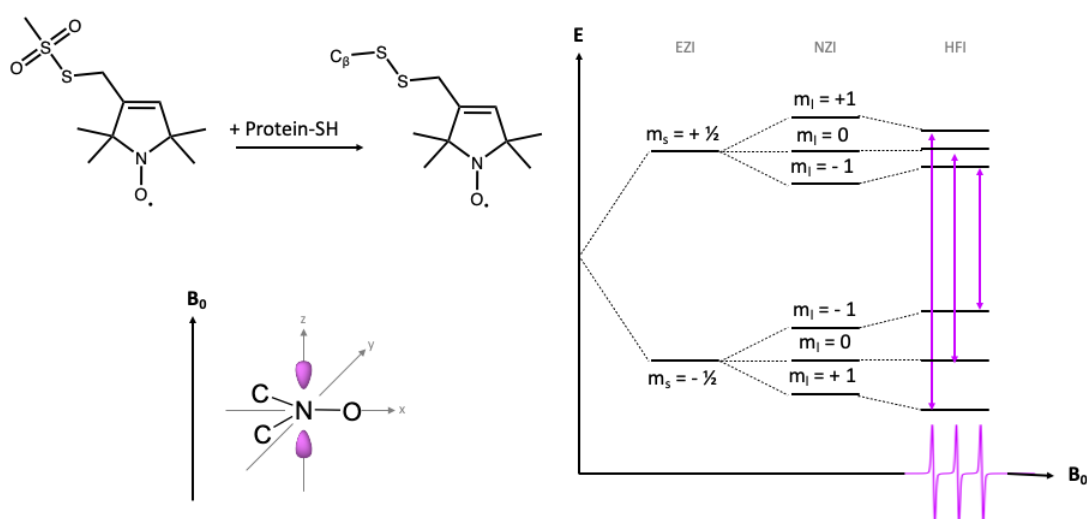


Figure 2.2 The chemical structure of the spin label, MTSL, which reacts specifically with cysteine residues on protein molecules to form a disulfide bond. MTSL contains a lone electron in the π orbital which is shown with respect to the cartesian axes and magnetic field, B_0 . The energy level diagram for a nitroxide radical species (such as MTSL) with $S = 1/2$ and $I = 1$ is shown with the electron zeeman interaction (EZI), nuclear zeeman interaction (NZI) and hyperfine interactions (HFI). An EPR spectrum is shown below resulting from the three allowed transitions (displayed as purple arrows).

2.1.3 Rotational correlation time

The rotational correlation time, or τ_c , refers to the mobility and rotational motion of the spin label and is arguably the most informative parameter. This is reflected in the EPR spectral line shape which can be used to gain information about the structure and environment of a protein. Molecules tumble in solution, unless they are frozen, and anisotropy is averaged to zero over time. This occurs for a free (fast) tumbling molecule which will give a narrow lined EPR spectrum with isotropic g- and A-values. However, if the tumbling is not fast enough in comparison to the intrinsic timescale of the EPR experiment ($\sim$ the Lamour frequency) and the anisotropy is not fully averaged out then broader spectral line shapes occur. The latter is the case for spin-labelled proteins. There are three main contributions: the rotational correlation time of the protein as a macromolecule in solution; the backbone motion of the protein at the position where the spin label is attached; and the rotational motion of

the spin-label side chain at the point of attachment.^{154,155} A spin label which is solvent exposed compared to a buried environment will present very different EPR spectral line shapes. Figure 2.3 shows EPR spectra with τ_c s varying between 0.1–15 ns. The EPR spectrum will be composed of the conformational states of the protein weighted by their relative proportions.

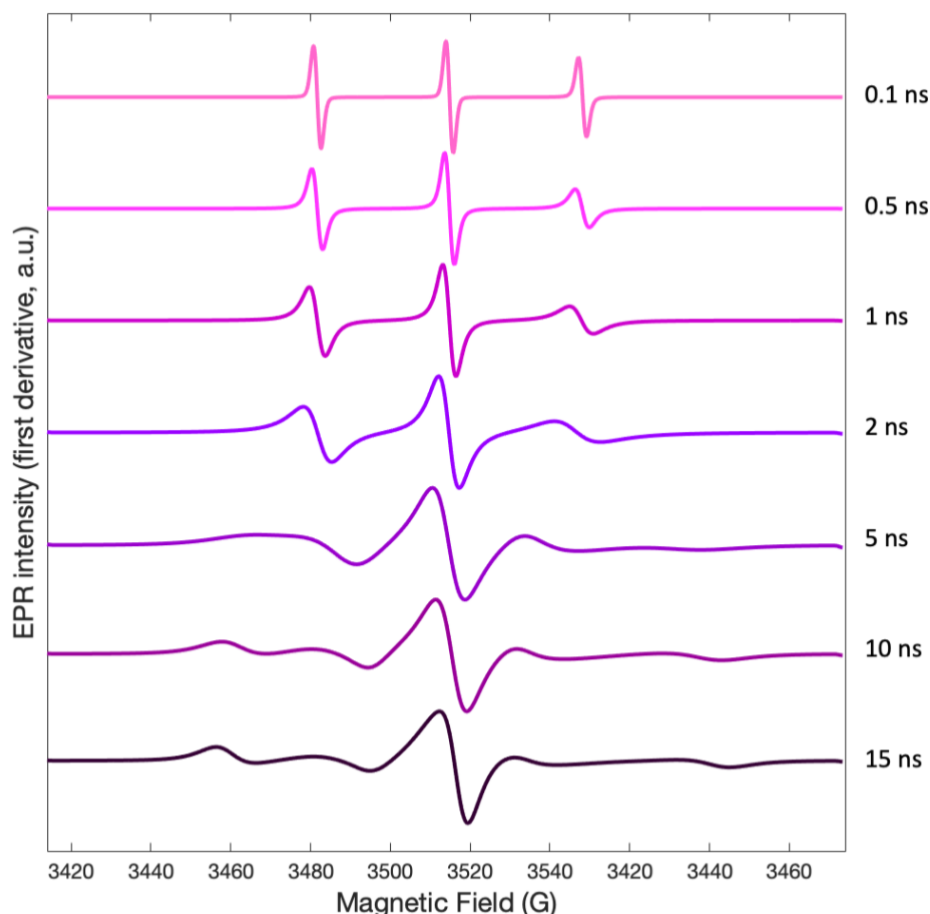


Figure 2.3 The effect of rotational correlation times on the spectral line shape of an EPR spectrum. Spectra were simulated using the EasySpin package within MATLAB for a typical nitroxide spin-label ($S = \frac{1}{2}$ and $I = 1$). The rotational correlation time for each spectrum is displayed on the right.

2.2 Fundamental theory

2.2.1 The spin Hamiltonian

The spin Hamiltonian is used to describe the energy of a spin system, simplifying the quantum mechanical description of EPR spectroscopy. The Hamiltonian, $\hat{H}$, is an

energy operator which can be broken down into the different physical phenomena contributing to the total energy of each state of the system (Equation 2.4).

$$\hat{H} = \hat{H}_{EZI} + \hat{H}_{NZI} + \hat{H}_{HFI} + \hat{H}_{EEI}$$

2.4

The contributions include the electron Zeeman interaction (EZI), nuclear Zeeman interaction (NZI), hyperfine interaction (HFI), nuclear quadrupole interaction (NQI) and electron-electron interaction (EEI).^{150,151}

- EZI = the interaction between an unpaired electron and the applied magnetic field
- NZI = the interaction between nuclear spins of nuclei and the applied magnetic field
- HFI = the interaction between an unpaired electron and the nuclear spins of nearby magnetic nuclei
- EEI = if there are multiple unpaired electrons present and they interact with each other. In the case where they are strongly coupled it is called zero field splitting (ZFS) and when weakly coupled it is dipole-dipole and exchange interactions

2.2.2 The electron Zeeman interaction

The electron has $S = \frac{1}{2}$ with two possible orientations of $m_s = \pm \frac{1}{2}$. In the absence of a magnetic field, the two states have the same energy and are degenerate. When the electron is placed in a magnetic field, the degeneracy is lifted, and the electron spin aligns parallel or antiparallel to the z-axis of the applied magnetic field (Figure 2.4).

These states are also referred to as spin-up ($m_s = -\frac{1}{2}$ or β) and spin down ($m_s = +\frac{1}{2}$ or α) respectively. This is the electron Zeeman interaction.^{150,153} The degree these states split is directly proportional to the strength of the applied (external) magnetic field, B_0 , which is described using Equation 2.5.

$$\hat{H}_{EZI} = g_e \cdot \mu_B \cdot B_0 \cdot S$$

2.5

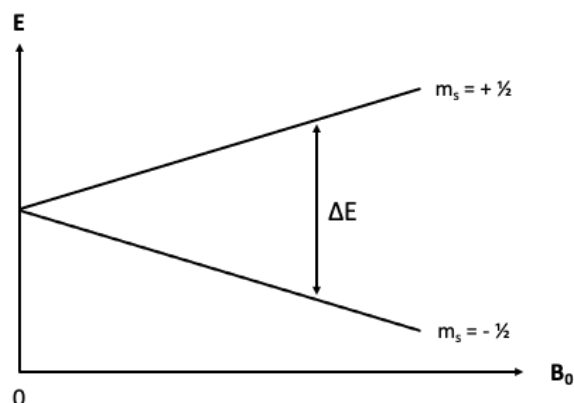


Figure 2.4 The electron Zeeman interaction shown for an $S = \frac{1}{2}$ system. In the absence of a magnetic field, B_0 , the energy levels are degenerate. This degeneracy is lifted when the electron is placed in a magnetic field causing splitting into two energy levels, $m_s = \pm \frac{1}{2}$ where the electron either aligns parallel or antiparallel to B_0 . Microwave radiation, $h\nu$, of a specific frequency will induce a transition between the two levels if the resonance condition is fulfilled. The energy gap, ΔE , between the two levels is proportional to magnetic field.

In order to observe an EPR signal, the resonance condition must be met. This is when the frequency of electromagnetic radiation (in the microwave region) matches the energy difference between the two states ($\pm \frac{1}{2}$) and causes transitions between them (Equation 2.6).

$$\Delta E = h\nu = g_e \cdot \mu_B \cdot B_0$$

2.6

Experimentally, a fixed microwave frequency is used, and the magnetic field is swept (separating the energy levels) between user defined limits. A signal is observed at each field value for which the resonance condition is satisfied. There are different 'bands' within the microwave region of the electromagnetic spectrum which are used for various EPR experiments. The names of these bands originate from WW2 radar use. The most common are X-band (~8–10 GHz), Q-band (~35 GHz) and W-band (~90 GHz).^{150,151}

EPR signal strength depends on the population difference between the two states, as described by the Boltzmann distribution (Figure 2.5 & Equation 2.7): where N

represents the population of the upper and lower energy levels, k_b is the Boltzmann constant ($1.3806 \times 10^{-23} \text{ J K}^{-1}$) and T is temperature. Absorption of microwaves results in the excitation of electrons from lower to higher energy levels within the ground state spin projection manifold, which in the absence of efficient relaxation pathways would result in the population of these levels moving away from thermal equilibrium with the surrounding lattice. These relaxation pathways include both the emission of photons with identical energy to those absorbed, imposing the requirement for a population difference in order for net absorption to be observed, and non-radiative routes in which energy is transferred into lattice vibrations.

$$\frac{N_{upper}}{N_{lower}} = e^{-\frac{\Delta E}{k_b \cdot T}}$$

2.7

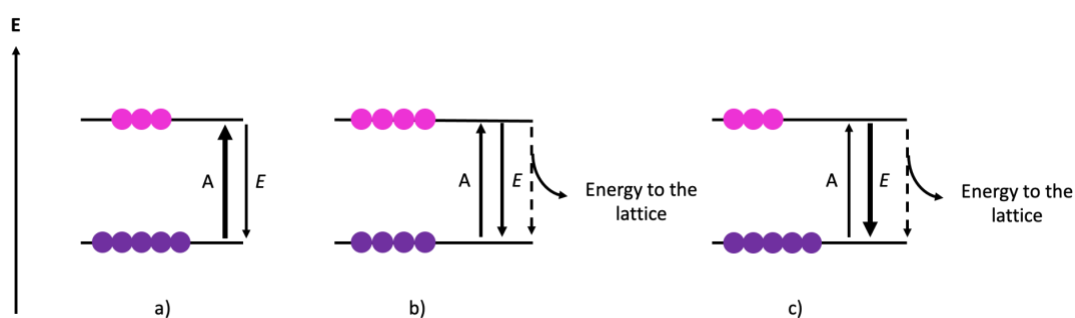


Figure 2.5 The electron spin in a magnetic field with the population of the upper and lower spin levels displayed as purple and pink dots respectively. The relative strength of absorption, A , and emission, E , are shown by the thickness of the arrows. A) At thermal equilibrium $A > E$ because of the population difference. B) When the system is saturated $A = E$ due to the equal population of the two levels. C) To re-establish the population difference energy is transferred via spin lattice relaxation (dotted arrow).

2.2.3 Spectroscopic g-factor

The g -value is a proportionality constant, which is 2.0023 for a free electron. However, when an unpaired electron is in an atom or a molecule, deviation from this value occurs due to the influence of the chemical environment. The spectroscopic g -value of a sample is commonly measured using a standard such as 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) or weak pitch which have g -values of 2.0036 and 2.0028 respectively.¹⁵⁰

In molecules, unless the unpaired electron is in an s-orbital, the orbital motion around the atomic nucleus gives rise to an orbital angular momentum, L . To account for this additional component, Equation 2.5 is adapted to give Equation 2.8.^{150,157} The electrons orbital angular momentum, like electron spin, has an associated magnetic moment. However, unlike S , it is anisotropic which means it is dependent on orientation within the external magnetic field.

$$\hat{H}_{EZI} = g_e \cdot \mu_B \cdot B_0 \cdot (L + S) \quad 2.8$$

The interaction between L and S is termed spin orbit coupling (S'). This causes g values to deviate from g_e and are referred to as the effective g -value, g_{eff} - resulting in Equation 2.9.¹⁵⁰

$$\hat{H}_{EZI} = g_{eff} \cdot \mu_B \cdot B_0 \cdot S' \quad 2.9$$

The situation becomes more complex when considering g -value anisotropy. Within the spin Hamiltonian description, the g -value is not a simple scalar quantity independent of magnetic field. It is broken down into a 3 x 3 g -tensor matrix which includes the three cartesian axes x , y and z (Equation 2.10). In diagonal matrices, all off-diagonal components are zero (i.e. g_{xy} , g_{xz} , g_{yx} , g_{yz} , g_{zx} , g_{zy}) leaving the diagonal components of g_{xx} , g_{yy} and g_{zz} - the principle g -values.⁴

$$g = \begin{bmatrix} g_{xx} & g_{xy} & g_{xz} \\ g_{yx} & g_{yy} & g_{yz} \\ g_{zx} & g_{zy} & g_{zz} \end{bmatrix} \quad 2.10$$

In EPR there are three different descriptions for a system: isotropic, axial, and rhombic - each of which are dependent on symmetry with respect to the x , y and z axes. By convention the axis with the highest symmetry is defined as z . If a frozen (solid) sample has symmetry where all three cartesian axes are equivalent, it is termed isotropic. In this case $g_{xx} = g_{yy} = g_{zz}$ and Equation 2.11 can be used to assign an Isotropic g -value, g_{iso} , by averaging the three components.^{150,157} This is rarely observed for solid samples but is more likely to be used in solution state where

molecules are tumbling rapidly in solution. As a result, their orientation with respect to the cartesian axes is lost and can therefore be described by a single value, g_{iso} . The EPR spectrum appears as a single first derivative peak (Figure 2.6).

$$g_{iso} = \frac{g_{xx} + g_{yy} + g_{zz}}{3}$$

2.11

Axial symmetry occurs when the x and y axes are equivalent to each other but not with z, resulting in $g_{xx} = g_{yy} \neq g_{zz}$. This case is also referred to as g-parallel ($g_{||}$) for g_{zz} and g-perpendicular ($g_{\perp}$) for g_{xx} and g_{yy} .¹⁵⁷ The resulting EPR spectrum has a single first derivative peak representing $g_{\perp}$ and a negative peak representing $g_{||}$ as the resonance condition is now fulfilled at different magnetic field values due to the orientational dependence. If $g_{||} > g_{\perp}$, the spectrum has a positive peak representing $g_{\perp}$ and a single first derivative peak for $g_{||}$. The final description is rhombic, where there is no symmetry between any of the axes resulting in inequivalent g-values ($g_{xx} \neq g_{yy} \neq g_{zz}$).¹⁵⁷ The rhombic spectrum has a positive peak for g_{xx} , first derivative peak for g_{yy} and a negative peak for g_{zz} in the convention of $g_{xx} > g_{yy} > g_{zz}$ (Figure 2.6).

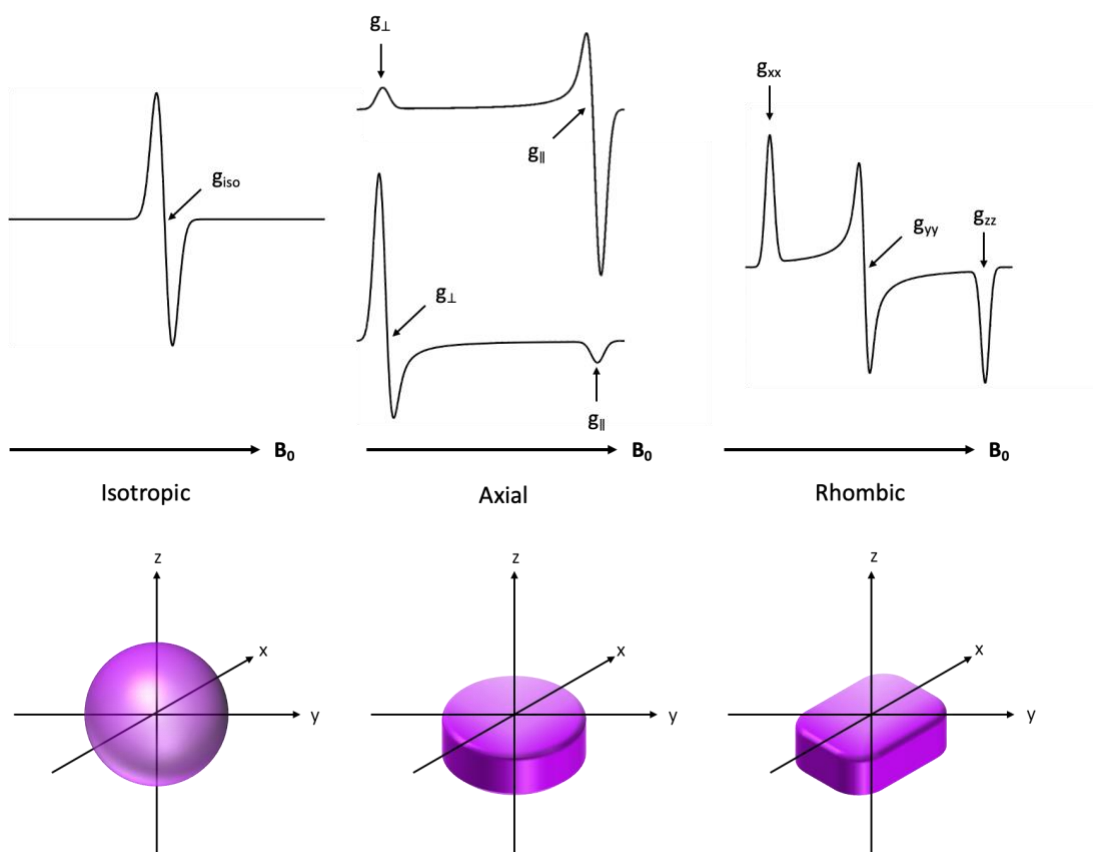


Figure 2.6 Three types of symmetry descriptions for an $S = \frac{1}{2}$ system: isotropic, axial and rhombic. The corresponding (frozen) EPR spectra are displayed (top) with the respective g -values shown for each. Axial is shown for when $g_{\perp} < g_{\parallel}$ (top) and $g_{\perp} > g_{\parallel}$ (bottom). These were simulated using the EasySpin package within MATLAB for an $S = \frac{1}{2}$ system. The description of each symmetry is shown at the bottom on cartesian axes.

2.2.4 The nuclear Zeeman interaction

The nuclear Zeeman interaction is used to describe the interaction between the non-zero nuclear spins (of nuclei) and the external magnetic field. Like electron spin, nuclear spin is characterised by the nuclear spin quantum number, I . Which in turn has an associated magnetic dipole moment, μ_I . Two examples of species with non-zero nuclear spins are protons ($I = \frac{1}{2}$) and ^{14}N nuclei ($I = 1$).¹⁵⁰ The nuclear Zeeman interaction causes splitting into $2I + 1$ levels which are described by the magnetic quantum number m_I , where $m_I = -I, -I+1, \dots, I-1, +I$. For example, $I = \frac{1}{2}$ causes splitting into 2 levels which are labelled $m_I = -\frac{1}{2}$ and $+\frac{1}{2}$ (Figure 2.7). Degeneracy of these states is lifted upon application of the external magnetic field, giving rise to the nuclear Zeeman effect. The energy of these levels is given by Equation 2.12 - where

g_N is the effective nuclear g-factor and μ_N is the nuclear magneton.¹⁵¹ Depending on the nucleus g_N can be positive or negative. The effect of the nuclear Zeeman interaction is smaller than that of the electron Zeeman interaction (Figure 2.7). For nuclei to be studied using NMR spectroscopy, they must have non-zero nuclear spin quantum numbers and transitions must obey the selection rule for NMR spectroscopy: $\Delta m_I = \pm 1$.

$$\hat{H}_{NZI} = -g_N \cdot \mu_N \cdot B_0 \cdot m_I$$

2.12

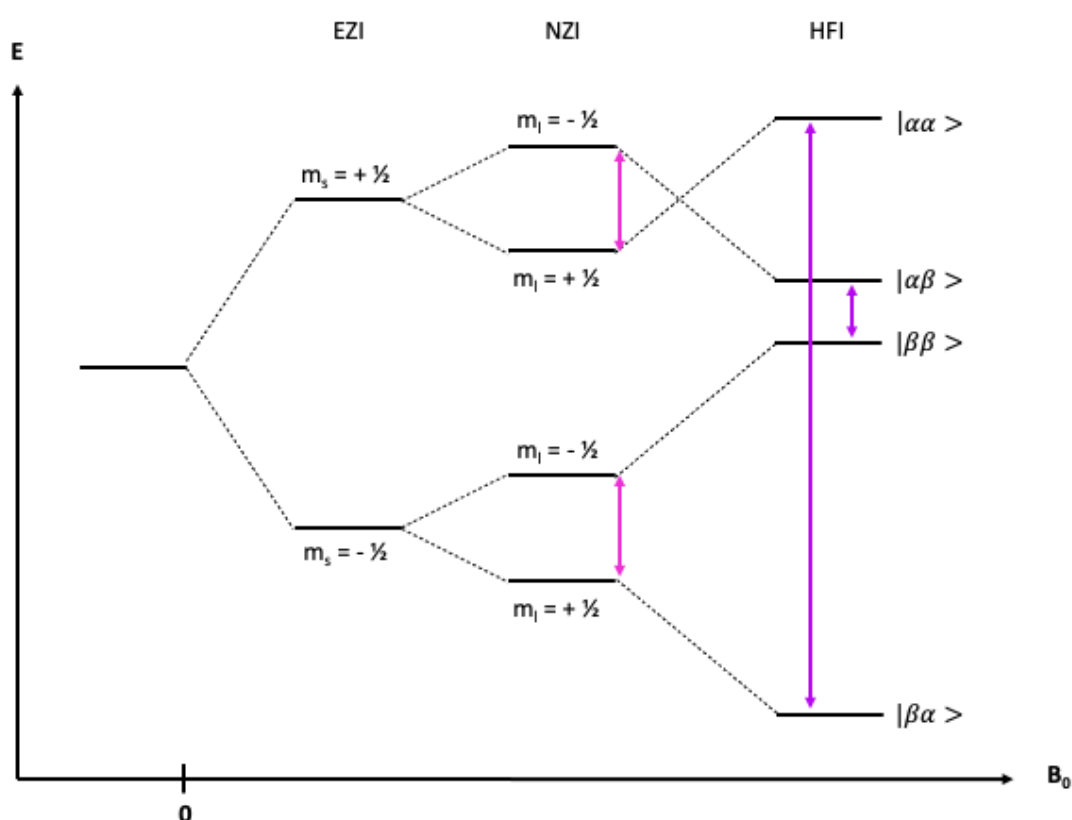


Figure 2.7 Energy level diagram of an electron ($S = 1/2$) interacting with a proton ($I = 1/2$). Splitting is caused by the electron Zeeman interaction (EZI), nuclear Zeeman interaction (NZI) and the hyperfine interaction (HFI). The allowed EPR transitions are shown as purple arrows which follow the selection rules of $\Delta m_s = \pm 1$ and $\Delta m_I = 0$. Allowed NMR transitions are shown as pink arrows following the selection rules $\Delta m_s = 0$ and $\Delta m_I = \pm 1$.

2.2.5 The hyperfine interaction

So far, we have considered the interaction of the electron and the nucleus with the external magnetic field. If the electron and nearby nuclei did not interact with each other, a very simple EPR spectrum would result - a single line centred at g_{eff} in the case of isotropic g . However, in reality, the unpaired electron does interact with the nearby magnetic nuclei and this is called the hyperfine interaction. This causes further perturbation to the energy level diagram by moving the nuclear energy levels to a higher or lower energy (Figure 2.7). The extent of perturbation is described by the hyperfine splitting tensor, A .¹⁵⁰ As a result of both the electron and nuclear Zeeman interactions, four energy levels are formed for an $S = \frac{1}{2}$ and $I = \frac{1}{2}$ system. There are two allowed EPR transitions according to the selection rules $\Delta m_s = \pm 1$ and $\Delta m_I = 0$.

The hyperfine Interaction has both Isotropic and anisotropic contributions where the latter has directional dependency in a magnetic field. For the isotropic hyperfine interaction, it is mostly applicable for the region inside the nucleus, relying on the finite probability of finding electron spin density inside.^{150,157} This is only valid for s-orbital electrons or those with partial s-character where the nuclear magnetic dipole is constant in all directions (so independent of magnetic field). In this case, the hyperfine coupling constant is referred to as A_{iso} . The anisotropic hyperfine interaction is used for the region outside the nucleus and is described by the anisotropic hyperfine coupling constant A_{ansio} . This is a classical dipole-dipole interaction which is dependent on both the orientation and distance between the electron and nucleus in question.^{150,157} The nuclear magnetic moment has its own local magnetic field (B_{local}) which can alter the external magnetic field, either by adding or subtracting, to form an effective magnetic field (B_{eff}). B_{eff} is dependent on the angle between the applied magnetic field and the vector between the two dipoles. The hyperfine interaction can be described using Equation 2.13. Like g -values, A also has three principal components of A_{zz} , A_{xx} and A_{yy} .

$$\hat{H}_{HFI} = I \cdot A \cdot S$$

2.13

2.2.6 Electron dipole-dipole interactions

If two or more unpaired electron spins interact it is called an electron dipole-dipole interaction. This is stronger than the interaction between an electron and a nucleus however it is still a weak interaction. Electron dipole-dipole interactions can be used to get information about the distance and orientation of the electrons involved, and therefore gives an insight into the overall system. This interaction is described using Equation 2.14 where S_A and S_B are the interacting electron spins and D is the electron dipole-dipole coupling tensor.

$$\hat{H}_{dip} = S_A \cdot D \cdot S_B$$

2.14

$$D = \frac{\mu_0}{4\pi\hbar} \cdot \frac{g_A g_B \mu_B^2}{r_{AB}^2} \cdot \begin{bmatrix} -1 & 0 & 0 \\ 0 & -1 & 0 \\ 0 & 0 & 2 \end{bmatrix} = \begin{bmatrix} -\omega_{dd} & 0 & 0 \\ 0 & -\omega_{dd} & 0 \\ 0 & 0 & 2\omega_{dd} \end{bmatrix}$$

2.15

Equation 2.15 is used to calculate D which includes the distance vector between the coupled spins A and B (r_{AB}), vacuum permeability (μ_0), the g-values of each spin and the dipolar coupling frequency (ω_{dd}) which can be expressed using Equation 2.16.¹⁵⁸ Here θ represents the angle between the distance vector r_{AB} and the external magnetic field.

$$\omega_{dd} = \frac{g_A \cdot g_B \cdot \mu_B^2 \cdot \mu_0}{4 \cdot \pi \cdot \hbar} \cdot \frac{1}{r_{AB}^3} \cdot (1 - 3 \cos^2 \theta)$$

2.16

The anisotropic dipole-dipole interaction is dependent on both the distance and orientation of the interacting unpaired electrons. If the distance is between 0.8–2 nm broadening of the EPR spectrum occurs as a result of the dipolar coupling. Pulsed EPR techniques are used for longer range distance determination using dipolar coupling interactions which are discussed in more detail in Section 2.3.5.

2.2.7 Multifrequency EPR

The majority of this thesis uses X-band continuous wave and pulsed EPR spectroscopy. However, a study using Q-band continuous wave EPR spectroscopy was used to demonstrate the increased resolution which can be achieved at higher frequencies. At room temperature X-band, only the isotropic g - and A -values are observed. If the temperature is reduced, more resolution is achieved but this only extends to resolve A_{zz} and g_{zz} . The more polar the environment of the spin label, the higher the A_{zz} and lower the g_{xx} value.¹⁵⁹ Moving to Q-band frequencies begins to resolve the x and y components and therefore hyperfine coupling constants can be distinguished. The most resolved microwave frequency is at W-band where better resolution of all x , y and z components can be achieved (Figure 2.8).

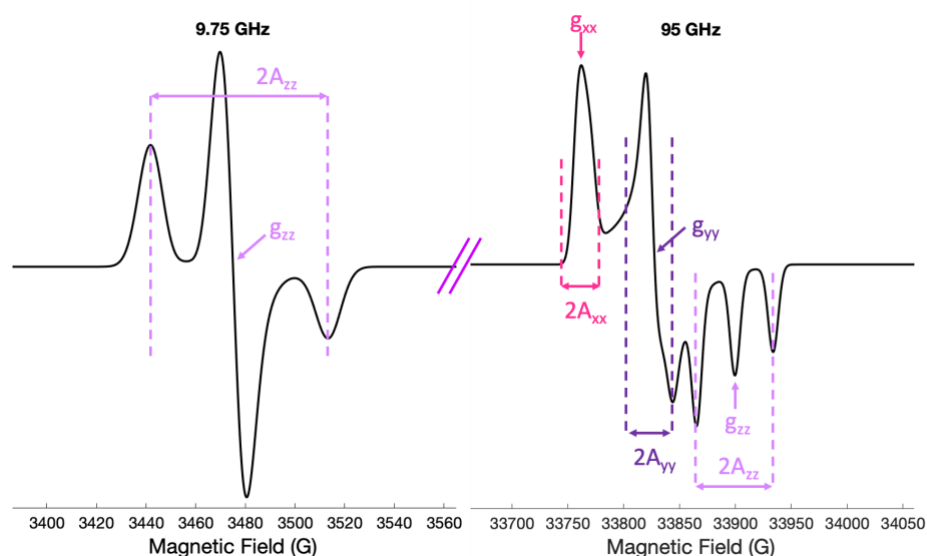


Figure 2.8 A (frozen) nitroxide spectrum at two different microwave frequencies: X-band (9.75 GHz) and W-band (95 GHz). The increased resolution obtained at higher frequencies (and field) has been shown by labelling g - and A -values which can be distinguished from each. Spectra were simulated using the EasySpin package within MATLAB.

2.3 Pulsed EPR spectroscopy

Both cw and pulsed EPR spectroscopy require a static field, B_0 , to magnetise the sample. In cw-EPR the sample is constantly irradiated at low power whilst the microwave field is swept, and the intensity of the reflected microwaves from the

cavity is recorded. In pulsed EPR, a sequence of short, high power pulses are used to manipulate the magnetisation away from Boltzmann distribution. Relaxation of magnetisation back to equilibrium is monitored via the induced current in the detector coils. In pulsed EPR, the resonator is over coupled and parameters in addition to magnetic field are varied (time delay, pulse lengths and/or frequency).¹⁶⁰

2.3.1 Reference frames, magnetisation, and pulses

Magnetisation is the ensemble of identical, non-interacting electron spins which can be described by the sum or average of the electron spin moments. Magnetisation behaves like a classical vector which has the cartesian axes (x, y, z). In EPR B_0 sits along the z-axis with microwave magnetic field B_1 perpendicular on the x-axis. If an electron is placed in B_0 it will experience a torque which is a measure of force that causes an object to rotate about an axis. In this case, the electron will precess about the z-axis (Figure 2.9). The frequency of this precession is described by the Larmor frequency, ω_L (Equation 2.17).^{151,160}

$$\omega_L = -\gamma \cdot B_0 \quad 2.17$$

Where γ is the gyromagnetic ratio (2.8 MHz/G for the free electron) and can be defined using Equation 2.18.

$$\gamma = \frac{-g_e \cdot \mu_B}{\hbar} \quad 2.18$$

It is energetically more favourable for spins to be in a parallel orientation along the z-axis. However, in the x-y plane they adopt random orientations. This means that the x-y components of the spins precessing about the z-axis will effectively cancel each other out to achieve stationary magnetisation, M_0 , along B_0 (Figure 2.9).¹⁶⁰

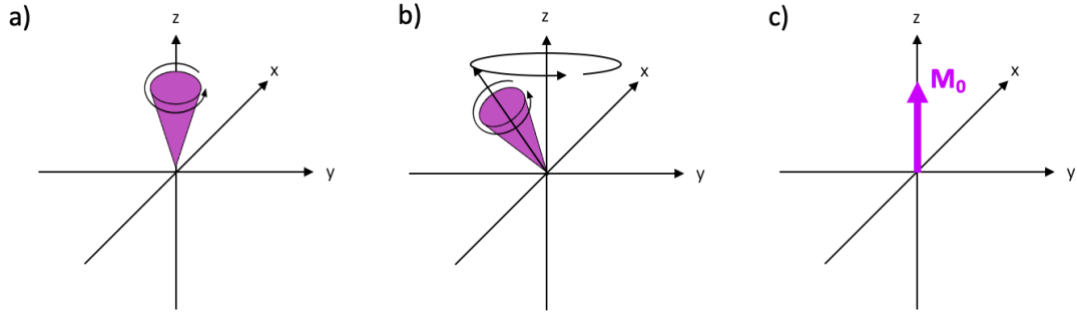


Figure 2.9 The movement of an electron placed in a magnetic field and the resultant stationary magnetisation a) The precession of a spin when parallel to the + z-axis, along which magnetic field is applied b) The precession of spin as a result of torque at an angle to the z axis c) A simplified representation of the net magnetisation vector, M_0

The rotating frame must now be taken into consideration. Resonators make linearly polarised microwaves which are equivalent to two magnetic fields rotating in opposite directions at the microwave frequency.¹⁶¹ This is what creates B_1 perpendicular to B_0 . The rotating coordinate frame turns at this frequency too so the microwave frequency ω_0 equals ω_L . The rotating frame makes the magnetisation at the Larmor frequency appear stationary as well as one component of B_1 , and the other component can be neglected. Only the effects of B_1 on M_0 need to be considered within the rotating frame since there is no precession. The rotation of the spins about B_1 occurs for the lifetime of the pulse and is defined by the Rabi frequency, ω_1 (Equation 2.19).^{151,160,161}

$$\omega_1 = \gamma \cdot B_1 \quad 2.19$$

If B_1 is parallel to the x-axis, the magnetisation vector M_0 is in the z-y plane rotating about the x axis. The angle by which M_0 is displaced from the z-axis is described by the tip angle, α . This can be calculated using Equation 2.20 where t_p is the pulse length (s).¹⁶¹

$$\alpha = -\gamma |B_1| t_p \quad 2.20$$

In practical terms, EPR experiments are labelled according to the tip angle with directionality from the axis they originate from. For example, $+x \frac{\pi}{2}$ pulse means when

the magnetic field is applied (B_1) along the + x direction, the $\frac{\pi}{2}$ pulse makes the spins rotate 90° into the -y axis where signal detection is aligned.^{160,162}

2.3.2 Free induction decay and off-resonances

Free induction decay (FID) is caused by the simplest of all pulse sequences - with a single pulse. The maximum FID amplitude occurs when the maximum number of spins are excited, which is caused by a $\frac{\pi}{2}$ pulse (equivalent to 90°). All spins with the same resonant frequency are termed a spin packet and start at thermal equilibrium. When a $\frac{\pi}{2}$ pulse is applied they will flip from the z plane into the x-y plane and the spin packet magnetic moments precess around the z-axis (in the x-y plane, Figure 2.10). The frequency of the precession of some spin packets (ω) is not equal to the microwave frequency (ω_0) which causes the rotation in the x-y plane. The frequency difference is expressed using Equation 2.21.¹⁶⁰

$$\Delta\omega = \omega - \omega_0$$

2.21

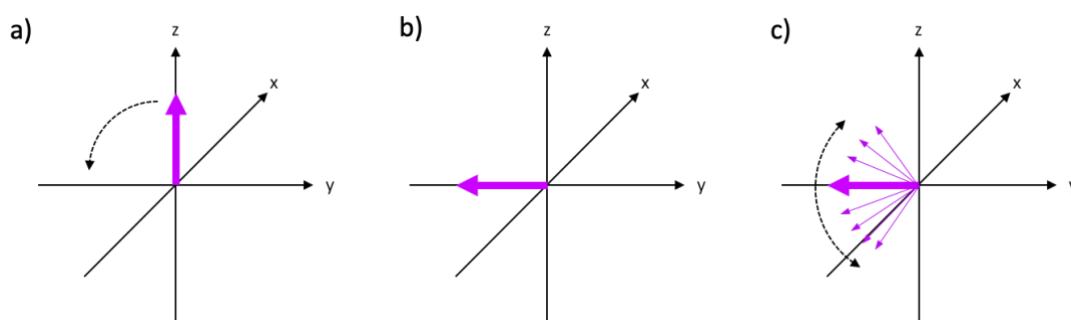


Figure 2.10 The bulk magnetisation vector (a) is tipped to the -y axis by a $\pi/2$ pulse (b). If the frequency of precession of the spin packets does not equal the microwave frequency, the magnetic moments precess around the z-axis in the x-y plane (c).

This precession about the z axis will occur at different speeds with some spins being faster than others. If $\Delta\omega > 0$ it results in anticlockwise precession and if $\Delta\omega < 0$ the spins will precess in a clockwise direction.¹⁵¹ The precessing total magnetic moment is the FID signal. The different precession frequencies of the spins cause the magnetic

moments to dephase. Over time, the magnetic moment in the x-y plane decreases as $\Delta\omega$ deviates from ω . The FID loses intensity until complete dephasing and relaxation has occurred. The effect of B_0 is no longer zero, so the magnetisation is tipped by the vector sum of B_1 and B_0 which results in an effective magnetic field, B_{eff} . Magnetisation is tipped around B_{eff} at a different effective rate, ω_{eff} (Equation 2.22).^{160,161}

$$\omega_{eff} = \sqrt{\omega_1^2 + \Delta\omega^2}$$

2.22

So overall the FID signal has 4 main properties: 1) occurs precisely at resonant frequency of the spin packet, 2) decays with relaxation of the spin packet 3) if the pulse deviates from $\frac{\pi}{2}$, the amplitude or phase may be affected but not the frequency or lifetime and 4) if the phase of excitation is shifted, the phase of the FID shifts by the same amount.^{151,160}

2.3.3 The primary two-pulse echo

In a primary two-pulse echo, the $\frac{\pi}{2}$ pulse is followed by a delay of duration τ for dephasing. Here spins give an FID and dephase as previously described with some gaining and some losing phase relative to the rotating frame.¹⁶⁰ After τ , a π pulse is applied which rotates all of the spin packets with respect to the y axis by 180°. This results in the spin packets with a higher precession frequency swapping with those that have a lower precession frequency. As a result, they all catch up with each other and refocus with -x phase at time τ after the second pulse (Figure 2.11). The emission signal is called a Hahn echo where the amplitude is proportional to the number of excited spins, like the FID. The maximum intensity is achieved using the $\frac{\pi}{2}$ and π pulse combination. This primary two-pulse echo forms the basis of many pulsed EPR sequences- one of which will be discussed in more detail later.

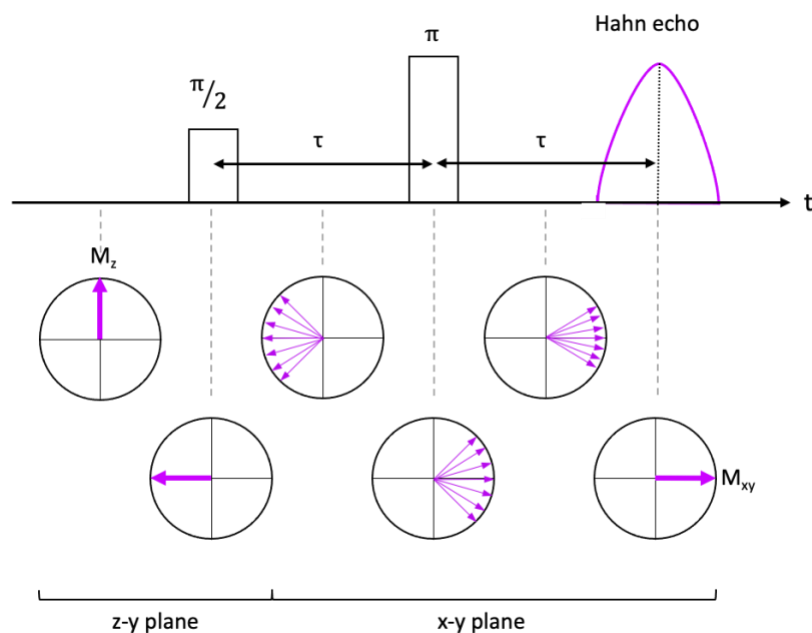


Figure 2.11 The primary two-pulse echo. A Hahn echo is created using a $\pi/2$ pulse, followed by a π pulse after time τ . The effect of the pulses on the magnetisation vector of spin packets is displayed with the viewing planes highlighted. This is also used for T_2 experiment.

2.3.4 Relaxation processes

When a pulse is applied and magnetisation is tipped into the x-y plane, it is a high energy state and relaxes back to the z-axis. The process of relaxation is split into spin lattice longitudinal relaxation, T_1 , and spin-spin transverse relaxation, T_2 .¹⁶¹ At thermal equilibrium, slightly more spins align parallel to B_0 in the + z direction in the lower energy state (as a result of Boltzmann distribution). In pulsed EPR to achieve zero net magnetization along the z-axis, a $\frac{\pi}{2}$ pulse is applied. Alternatively, if a π pulse is applied then the higher energy state will contain slightly more spins due to the inversion of the equilibrium populations. The net magnetisation moves from + z at thermal equilibrium to -z for the π pulse, hence it is called an inversion pulse. These three scenarios are displayed in Figure 2.12.

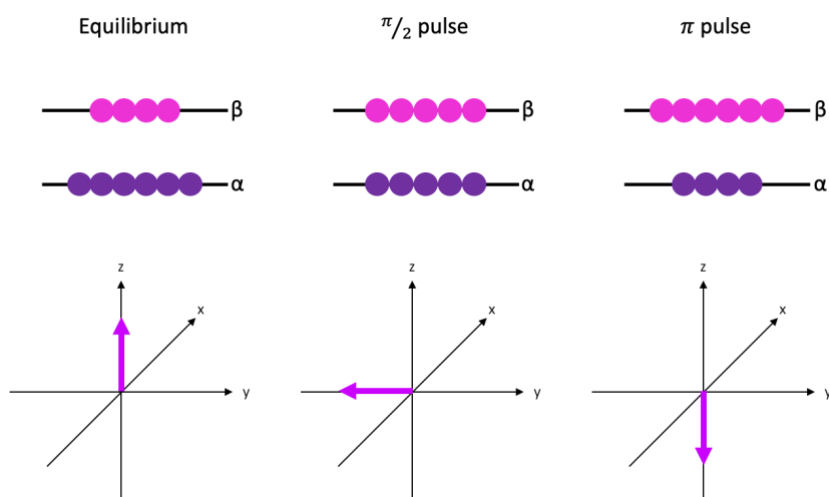


Figure 2.12 The effect of pulses on the population states of the two energy states for an $S = \frac{1}{2}$ system (α and β). At thermal equilibrium bulk magnetisation lies along the + z axis, along which the magnetic field is applied. Upon application of a $\pi/2$ pulse, in the x-y plane, magnetisation is shifted to align with the -y axis and the population of both energy levels is equal. If a π pulse is applied to a system in equilibrium, the magnetisation is inverted (to align with the -z axis) as well as inversion of the populations of both levels.

T_1 represents the time it takes for non-equilibrium magnetisation to relax back to thermal equilibrium along the z-axis (i.e. back to M_0 , Figure 2.13). All spins need to return to thermal equilibrium before the next pulse sequence can begin. Spin-lattice relaxation as its suggested by the name, occurs through interactions with surrounding atoms or molecules (the lattice). Experimentally T_1 limits the length of pulse sequences and the shortest possible repetition time. It can be measured experimentally using inversion recovery which is a three pulse sequence (Figure 2.14).^{160,161} The Hahn echo intensity is measured as a function of increasing time delay, T , between the inversion pulse and the detection sequence.

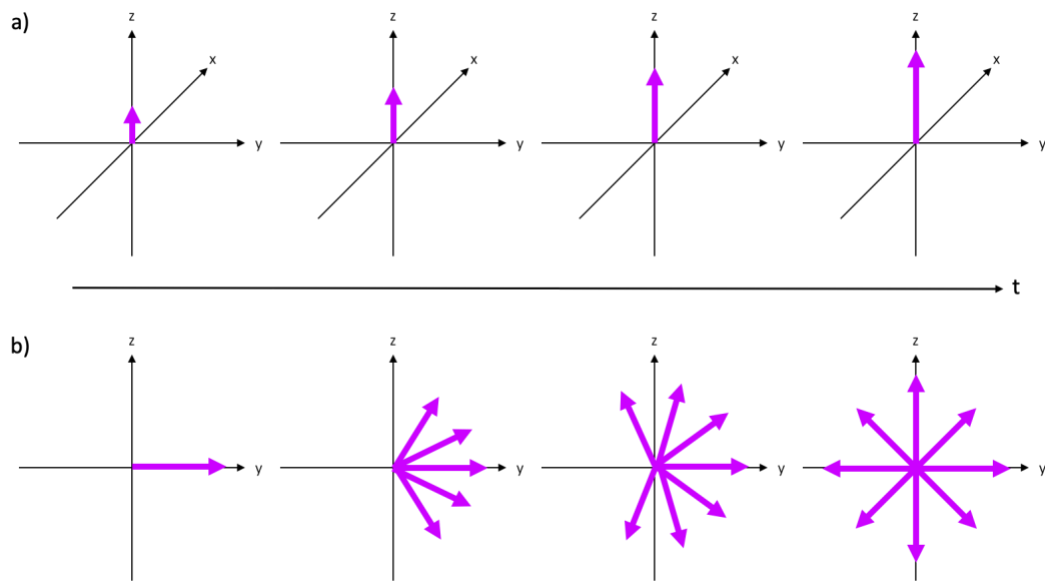


Figure 2.13 The two relaxation mechanisms (a) Spin lattice longitudinal relaxation (T_1) and (b) spin-spin transverse relaxation (T_2).

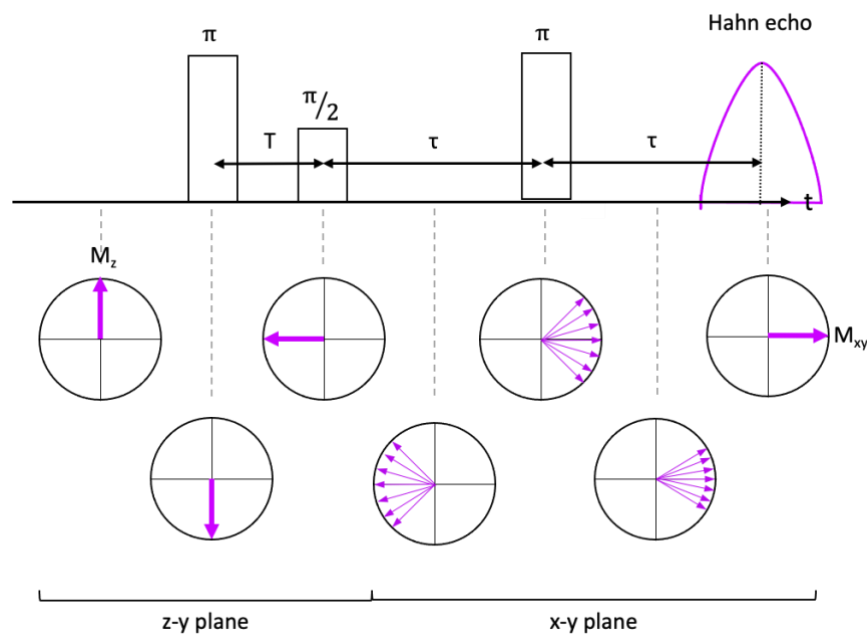


Figure 2.14 The pulse sequence for an inversion recovery experiment for T_1 relaxation time determination. The effect of the pulse sequence on the magnetisation vector of spins is shown with viewing planes highlighted.

T_2 is the time it takes for the spin packets magnetic moments in the x-y plane to lose phase coherence (where M_x and M_y are decaying to zero, Figure 2.13). It is always shorter than T_1 and is measured using a two pulse echo as a function of the time between two pulses in the detection sequence, τ . This is also referred to as a two

pulse echo spin echo envelope modulation (2P-ESEEM) experiment and has been described in Section 2.3.3. The two main contributions to T_2 are homogeneous and inhomogeneous broadening (Figure 2.15). For homogeneous broadening, spins experience the same magnetic field and interact which causes random spin ‘flip flops’. This results in a Lorentzian line shape which is the sum of a large number of lines with the same line width and Larmor frequency. Inhomogeneous broadening is when the spins experience slightly different magnetic fields due to unresolved hyperfine structure (i.e. unresolved splitting from nuclear and electron spins). The resulting EPR spectrum is a sum of several homogeneously broadened lines which are shifted in frequency, commonly resembling a gaussian line shape.¹⁶¹ For shorter T_2 , magnetisation dephases quicker in the x-y plane.

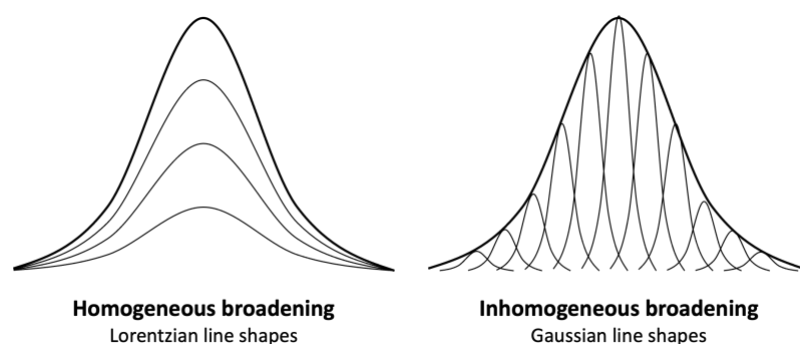


Figure 2.15 Homogeneous and inhomogeneous broadening

Relaxation time scales change with conditions such as temperature and concentration. In pulsed EPR experiments of spin-labelled protein described in this thesis, temperatures of 50 K were used. For each sample, the relaxation (T_2) profile was measured to optimise the experimental parameters of the pulse sequence for distance determination. This required long inter-pulse delays to detect longer distances. Optimising conditions to get the longest possible T_2 maximises the opportunity to detect low frequency oscillations corresponding to longer inter-spin distances in the pulsed EPR experiments (described in Section 2.3.5).

2.3.5 The double electron-electron resonance (DEER) experiment

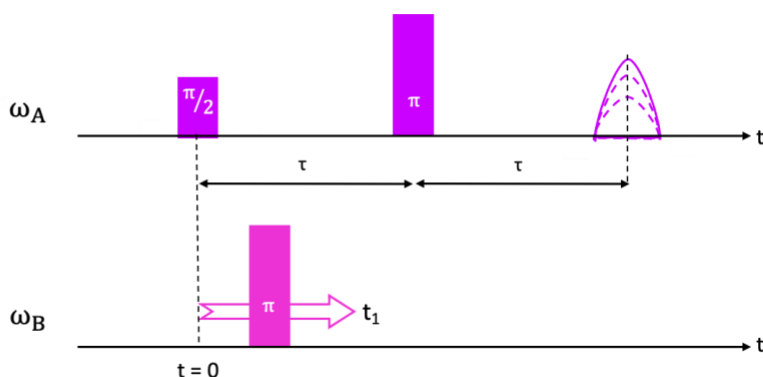
The double electron-electron resonance (DEER) experiment is used to obtain distances between two or more spin probes by measuring their dipole-dipole interaction. Typically distances between 2–8 nm can be measured for various samples including nucleic acids, peptides, soluble proteins, membrane proteins etc. This technique complements other structural biology methods such as x-ray crystallography, NMR spectroscopy and electron microscopy and can enable new minor population states to be detected.

After a pulse, the microwave power needs to dissipate and reach a safe level for the detector; the time it takes to do this is the deadtime of the spectrometer. Echoes or FID's cannot be detected during this time so deadtime can result in clipping¹⁶⁰. The original DEER sequence was a 3-pulse experiment which has since been developed into a 4-pulse method to eliminate the inherent dead time (Figure 2.16). The 3-pulse sequence was the original implementation of the DEER experiment but had pulse overlap between the first pulse ($\frac{\pi}{2}$) and pump pulse (π), causing artefacts around $t = 0$. Generation of a refocused echo in the 4-pulse experiment allowed accurate measurement around $t = 0$.¹⁶³ The 4-pulse DEER sequence has been used for all pulsed experiments in this thesis and is the most commonly used DEER method to date.¹⁶⁴ The 4-pulse sequence is measured across two different microwave frequencies where the perturbation by the pump frequency is monitored at the observe frequency. The interactions between the two spin probes are dependent on both spin-spin distance and the angle between the inter-spin vector and the applied magnetic field. The dipolar coupling, ω_{dd} , is defined using Equation 2.16 previously mentioned in Section 2.2.6.^{165,166}

The two coupled spins will be referred to as A and B. In a 4-pulse DEER experiment, a π -pulse is used to form the echo which informs on the modulation of the A-spins magnetisation by the dipolar coupling to the B-spins. The A-spins use the observe frequency, ω_A , and the B-spins use the pump frequency, ω_B . The overall pulse sequence in ω_A is: $\frac{\pi}{2} - \tau_1 - \pi - \tau_1 - echo - \tau_2 - \pi - \tau_2 - echo$. This includes a

standard echo detected EPR sequence to create the first (reporter) echo. Just before this echo, a π pulse in ω_B is applied at a variable time, t , as an inversion pulse which sweeps through the reporter echo. In ω_A , a π pulse at a time delay, τ_2 , is used to refocus the primary echo. The modulation in intensity of the refocused Hahn echo is recorded as a function of t (Figure 2.16).^{166,167}

3-pulse DEER sequence



4-pulse DEER sequence

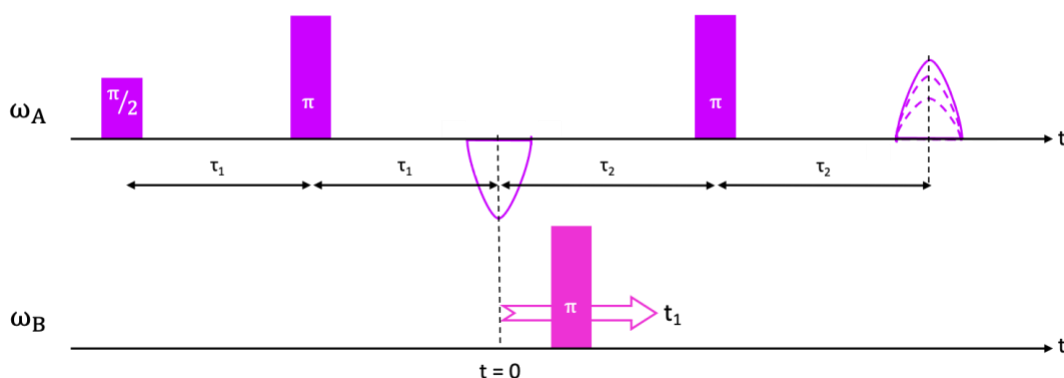


Figure 2.16 Schematic of the 3-pulse DEER sequence and the 4-pulse DEER sequence. The 4-pulse sequence eliminated deadtime and is most widely used. Two different frequencies are used: the observe frequency, ω_A , and the pump frequency, ω_B . The time delays between the pulses are detailed.

In terms of magnetisation, all spins start at thermal equilibrium along the + z axis. The $\frac{\pi}{2}$ pulse in ω_A flips bulk magnetisation of the A-spins into the x - y plane and dephasing begins (as a result of local field inhomogeneities). The π pulse applied after time τ_1 inverts the dephasing magnetisation by 180° so it refocuses, and a Hahn echo occurs. Note this Hahn echo is undetected. So far this is the typical Hahn echo sequence. The

π pulse in the second frequency, ω_B , is applied at a defined number of linearly spaced time points, t . This pulse flips the B-spins by 180° so they lie along the $-z$ axis. A second π pulse in ω_A refocuses the dephasing A-spins. If the two spins have dipole-dipole interactions, it changes the net angular precession rate of A-spins caused by phase lag. This means the dipolar coupled A-spins are not refocused and the Hahn echo intensity is affected. The extent of this depends on timings of the pump pulse, t_1 , and the dipolar frequency, ω_{dd} . The oscillating decay observed results from the Hahn echo intensity measured at each time increment the pulse pump is applied, t .¹⁶⁶

The refocused echo decay is controlled by the phase memory time constant, T_m . This needs to be considered to prevent signal loss as it limits the length of inter-pulse delays; the longer the inter-pulse delay, the greater the signal loss.¹⁶⁰ Data is recorded in the time domain. Longer distances have lower frequency oscillations, because ω_{dd} is inversely proportional to r_{AB}^3 as per Equation 2.16. Therefore, longer distances need a longer time to measure the refocused echo intensity. This is limited by the T_2 relaxation of A-spins as some magnetisation needs to be present to detect the A-spin Hahn echo.

Before the DEER experiment can be set up, a field sweep echo (FSE) is recorded to locate the optimal pump and observe frequencies. The FSE experiment measures the FID of the sample as a function of magnetic field, producing an absorption spectrum. One of the most common set ups for DEER experiments is to select the pump position as the point of maximum EPR intensity on the FSE spectrum, and the detect position on the low field edge which is the next highest point of signal intensity (Figure 2.17). The difference in magnetic field between the two points can be extracted and converted into frequency (by multiplying magnetic field in Gauss by 2.803). One key consideration is that overlap between the two frequencies due to the bandwidth of the pulses must be avoided. A nitroxide typically has a frequency difference of around 70 MHz for the aforementioned positions.

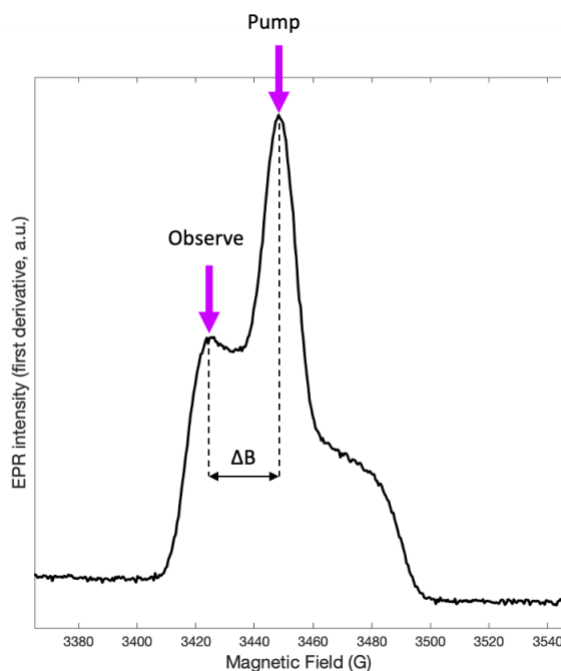


Figure 2.17 A field sweep echo (FSE) spectrum which is used to determine the optimal positions for pump and observe in the DEER experiment. The difference in magnetic field, ΔB , between the two positions is converted into a frequency and used in the DEER experiment set-up.

The second experiment which must be completed as part of the optimisation for the DEER experiment is the determination of phase memory time (the T_2 experiment). This looks at the phase memory relaxation rate of the sample using a Hahn echo sequence (Figure 2.11) where the echo intensity is measured as a function of time, τ . It is dictated by T_2 as this is the shortest of the two spin relaxation times. To extend the relaxation time lower temperatures can be used (50 K for a nitroxide) and deuteration which is typically achieved through the addition of d_8 -glycerol which also acts as a cryoprotectant. T_2 dictates the maximum delay time that can be chosen and therefore the upper limit on the distances that can be detected. This will be discussed in more detail in Section 4.1.

2.3.6 Interpreting the data

The most common method for interpreting DEER data is a MATLAB[®] based package called DeerAnalysis.¹⁶⁴ This has been developed into additional versions called DeerNet and most recently, DeerLab, which reduce the user bias when fitting and

interpreting the raw traces.^{168,169} In the time traces obtained from a DEER experiment, the presence of short distances can be observed as sharp decays and pronounced oscillations, whereas longer distances are less steep with less clear oscillations. The traces are a linear combination of oscillations with frequencies corresponding to each of the distances within the sample ensemble. If there are multiple species present in the sample i.e. several protein conformations, these observations may be less evident in the raw traces because all the different distances will contribute in their relative populations. Distance distributions are shown with colour-coded levels of certainty in the background ranging from green (reliable distance predictions) to red (data should not be interpreted). As the time the Hahn echo is measured for changes, the reliability of distances changes i.e. a longer τ means longer distances can be recorded with more certainty.

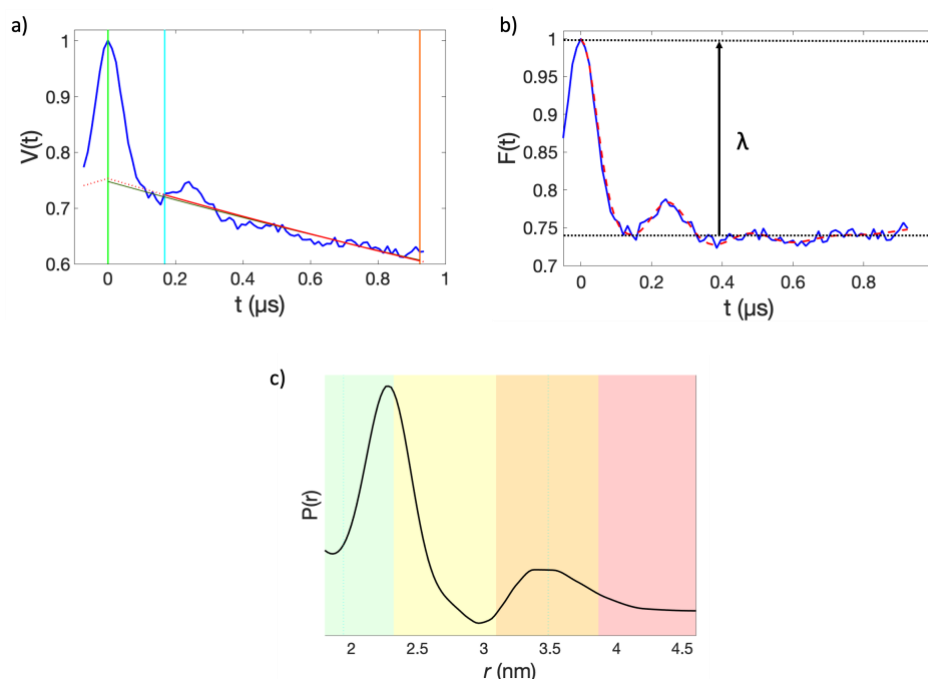


Figure 2.18 Processing DEER data using DeerAnalysis software within MATLAB. A) Primary data $V(t)$ (blue) with the fitted background function shown in red, where the dashed red line is the extrapolated background fit. The horizontal lines represent the zero time determined using intensity maximum (green); optimal starting time for background fitting determined by minimising probability density at the maximum distance (light blue); data is cut to minimise influence of artefacts (orange). B) The form factor $F(t)$ (blue) and its fit (red dashed) obtained by background fitting primary data with variable background models C) Resultant distance distributions obtained by Tikhonov regularisation. Background colours indicate the reliability of the results based on primary data: reliable (green), mean distances reliable and their widths (yellow), only mean distances reliable (orange), data shouldn't be interpreted (red).

Spectra have an intermolecular contribution which is the background factor or exponential decay.¹⁶⁴ The form factor is also contained within the spectra as the oscillations resulting from the interactions of the two spins. During the DeerAnalysis process (Figure 2.18), the exponential decay is fitted using the background function and subtracted to resolve the form factor. The modulation depth (λ) of the spectrum includes what the form factor decays to indicating the number of interacting spins. The background subtracted spectrum is converted into a distance using Tikhonov regularisation.

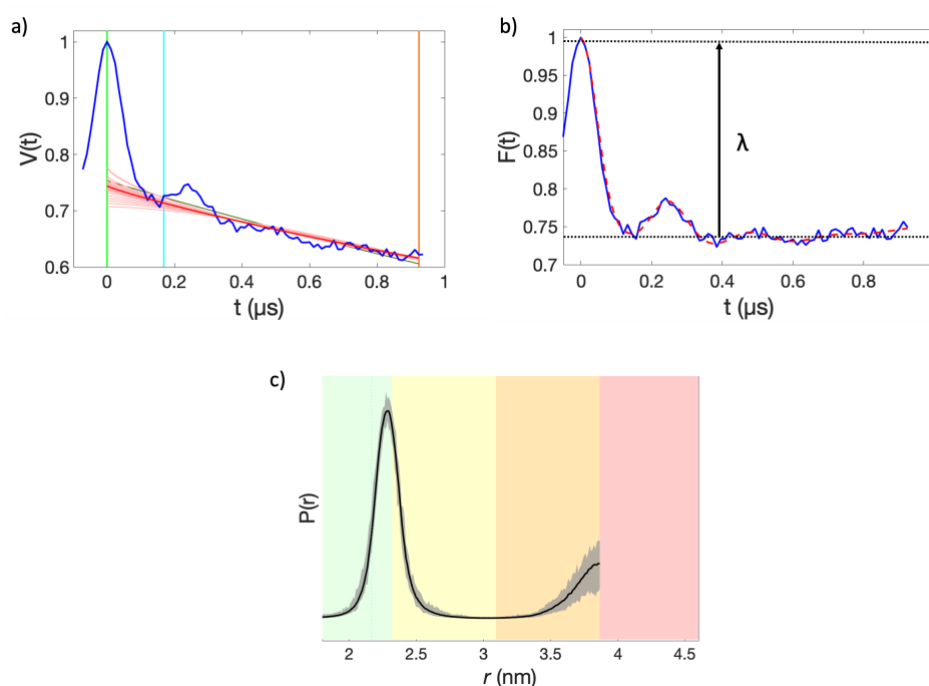


Figure 2.19 Analysis of DEER data using DeerNet. A) The primary data $V(t)$ (blue) with the fitted background function shown in red. There are more dashed red lines compared to DeerAnalysis which represent the extrapolated background fit. DeerNet software selects the background as opposed to DeerAnalysis by the user. B) The form factor $F(t)$ (blue) and its fit (red dashed) obtained by background fitting primary data with variable background models C) Distance distributions calculated using DeerNet are shown with 95% confidence bounds (grey). Background colours still shown to indicate the reliability of the results based on primary data.

Tikhonov regularisation is a proven method to produce clear and interpretable distance distributions when applied correctly. However, for datasets which are featureless, or when applied incorrectly, it can result in overinterpreted data.¹⁶⁸ This led to the development of DeerNet which includes the calculation of uncertainty in the distance distributions, taking away the need for the user to distinguish between

real distances and artefacts (Figure 2.19). The software uses a neural network which has been trained to solve spectra to obtain distance distributions with coloured confidence bands. DeerNet calculates the background function, instead of being manually selected in DeerAnalysis, helping to remove user bias and manipulation of data. For all interpreted DEER data in this thesis, DeerNet has been used to obtain distance distributions.

2.4 Fundamental theory of protein NMR spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy is a useful tool in structural studies but also to investigate phenomena such weak intermolecular binding events with K_D values in the μM – mM range.¹⁷⁰ NMR spectroscopy can be used to investigate biological binding processes at a molecular level for systems such as hormones and receptors, or proteins and gene regulators. There are two main approaches to this: either focusing on the protein resonances or ligand resonances. For fragment based screening the ligand based approach is typically employed to screen hits, followed by a protein-based method for validation of the hits.¹⁷⁰ The main changes observed during binding process are in chemical shift, relaxation times, nuclear Overhauser effects (NOE's) and exchange of magnetisation. The NOE is when spectral intensities of a spin changes as a result of the saturation of a nearby spin whose resonance has been perturbed.^{171,172}

As with any method involving membrane proteins there are limitations due to the low yields obtained and concentrations required. There is also the notorious issue of stability and function of membrane proteins and the presence of a membrane-like environment or lack thereof, for physiologically relevant results. NMR spectroscopy can be applied to samples in either a solid or liquid state depending on the experiment being used. Both of these techniques come with their own advantages and disadvantages. Solution state NMR is sensitive to molecular tumbling and so for membrane proteins it restricts the environment the protein can be studied in. This is generally limited to detergent micelles, small bicelles and nanodiscs.¹⁷³ There are also size limitations for protein-focused NMR experiments, with relatively small molecular

weight limitations for structure determination.^{173,174} The size limit has been pushed to ~100 kDa, but routine measurements typically reach 30 kDa, where small sized membrane proteins are <150 kDa.^{26,175,176} In addition, relaxation is dependent on molecular tumbling and can affect the sensitivity and resolution of experiments. Larger proteins have slower molecular tumbling which leads faster relaxation, as a result of increased spin-spin interactions. This causes decreased spectral intensity and resolution^{177,178} The increased line width associated with slower tumbling and larger number of signals (which increase with molecular weight) can cause resonance overlap making interpretation difficult. Pulse sequences in solid state NMR (ss-NMR) are less sensitive to the relaxation as the sample is mechanically spun at high speed.¹⁷³ Neither technique requires the attachment of exogenous labels in contrast to EPR spectroscopy and fluorescence techniques, but isotope labelling (such as ¹³C and ¹⁵N) can be used to study conformational changes (in solution) and reduce spectral crowding for ss-NMR.¹⁷³ Despite the limitations of protein NMR techniques, it is a promising tool for studying membrane proteins, particularly when used in combination with other biophysical methods such as X-ray crystallography and cryo-EM.

In NMR spectroscopy, a receiver coil detects the induced current from the precessing magnetisation and measures the free induction decay (FID, see Section 2.3.2), also referred to as the NMR signal. After the RF pulse, spins return to their equilibrium distribution as determined by the Boltzmann equation through relaxation (T_1 and T_2 described in Section 2.3.4), and the FID signal decays. The FID signal is Fourier transformed into the frequency domain where chemical shift (δ , in ppm) and relative intensity can be observed. Different nuclei resonate at different frequencies which are modified by the electronic environment to give multiple resonance frequencies, defined using the dimensionless chemical shift scale. This can be calculated using Equation 2.23 where ν is the frequency of the sample and ν_{TMS} is the frequency of the reference tetramethylsilane (TMS) used in ¹³C and proton NMR.

$$\delta \text{ (ppm)} = \frac{\nu - \nu_{TMS}}{\nu_{TMS}} \times 10^6$$

2.23

2.5 Saturation transfer difference (STD) NMR

Saturation transfer difference (STD) NMR is a ligand-based technique based on the NOE- a through space relaxation mechanism. This is a result of changes in the population states of coupled spins. Two nuclei with spin $\frac{1}{2}$ (S and I) which are coupled by a dipolar interaction will have four energy states (Figure 2.20). The frequency for the two I-spin transitions are the same, and the two S-spin transitions are the same. When the S-spins are saturated, the relative population of $\alpha_I\alpha_S = \alpha_I\beta_S$, and $\beta_I\alpha_S = \beta_I\beta_S$ where subscript letters represent the I and S-spins respectively.^{171,172} At thermal equilibrium the $\alpha\alpha$ and $\beta\alpha$ states contain a larger population of spins due to the Boltzmann distribution. However, when the S-spins are saturated, these states are now less populated and the $\alpha\beta$ and $\beta\beta$ spin populations are increased. The system returns to equilibrium following saturation through relaxation mechanisms. In this case, spin-lattice relaxation (w_1) has no effect on the population of spin I because the two transitions have the same relaxation rate. The other two relaxation mechanisms are the zero-quantum transition (w_0) and the double-quantum transition (w_2).

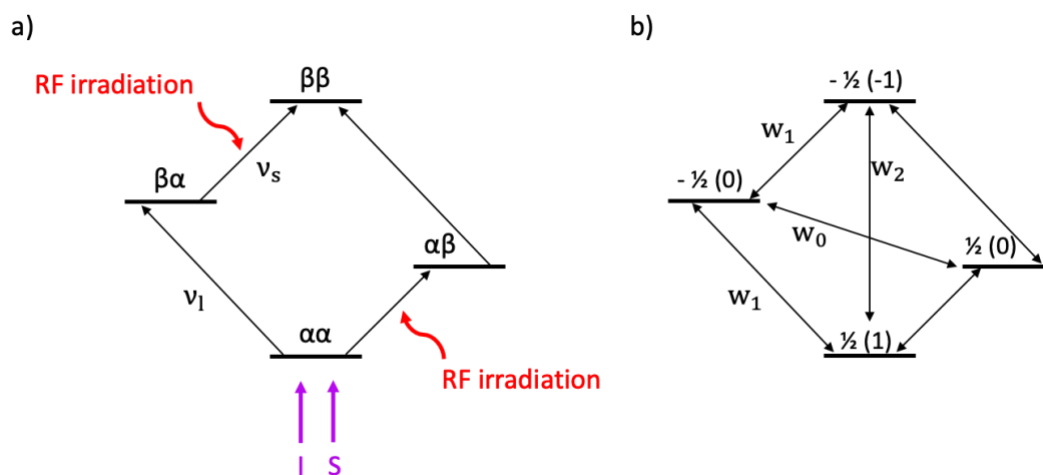


Figure 2.20 An energy diagram for a two-spin system (spin I and spin S). (a) The four energy states where the two S-transitions, ν_S are saturated by radiofrequency (r.f.) irradiation. These transitions are of equal frequency, as is $\alpha\alpha \rightarrow \beta\alpha$ and $\alpha\beta \rightarrow \beta\beta$ (ν_I). The first state represents spin I and the second spin S. (b) The zero-, single- and double- quantum transitions are shown by w_0 , w_1 and w_2 . The numbers represent the population of the state before (in brackets) and after saturation.

The zero- and double-quantum transitions are both forbidden by the NMR selection rules so will not be observed in an NMR spectrum. They are both types of cross relaxation which only occurs when the two spins are close together as it is caused by the exchange of magnetisation via dipole-dipole interactions between the two spins¹⁷¹. The result of this is a change in the intensity of spin I which can either increase (positive NOE) or decrease (negative NOE) depending on the correlation time of the molecule. Small molecules (MW <1000 Da), which tumble rapidly in solution, have dominant w_2 relaxation and exhibit positive NOE's.¹⁷⁹ The opposite is true for large molecules (MW >40 kDa), and for molecules of intermediate size the two relaxation mechanisms compete. When in complex with a protein the ligand tumbling rate is slowed to approximately that of the larger molecule and therefore exhibits the same NOE behaviour.¹⁷¹ As a result, if a ligand binds to a protein a large negative NOE is observed for the ligand. This is the basis of STD NMR where the ligand signal decreases when the protein is saturated (on-resonance experiment in Figure 2.21).

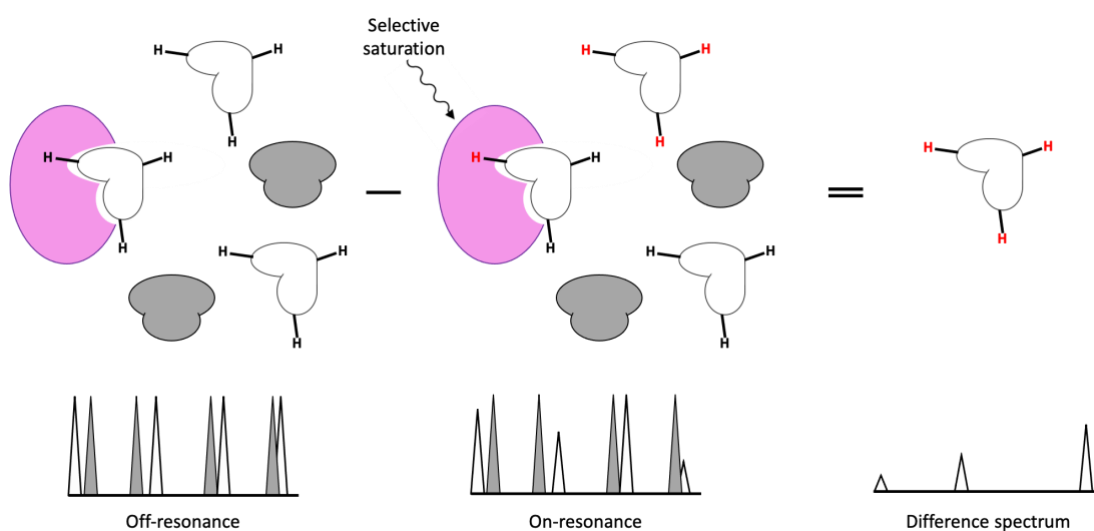


Figure 2.21 For an STD NMR experiment, an on- and off-resonance spectrum is recorded and the change between the two is displayed as a difference spectrum. This only shows resonances of ligand molecules (white) which have bound to the protein (pink) and received saturation (displayed in red). Non-binding ligand (grey) appears in both the on- and off-resonance spectra in the same intensity as no saturation is received, and therefore doesn't appear in the difference spectrum.

Since STD-NMR is a ligand-observe technique, it is not limited by protein size making it ideal for membrane proteins. The high sensitivity of this technique means it does not need high protein concentrations or isotopic labelling.¹³³ The main requirement is the presence of protein-ligand interactions i.e. binding and dissociation with K_D values in the μM – mM range.¹⁷⁰ STD NMR titration experiments can be used to calculate the dissociation constant for weak binding ligands which typically have K_D values between high nanomolar and low millimolar.^{133,172,180} If the K_D value of a weak binding ligand is known, a competition titration can be used to determine the K_D value of a higher affinity ligand extending the range of K_D 's that can be determined using STD NMR.^{133,180} An on-resonance NMR spectrum is recorded where the protein is selectively saturated at a resonance away from the ligand (usually around 1 to -1 ppm).^{133,172,180} In addition, an off-resonance spectrum is recorded where a frequency is selected away from both the protein and the ligand (>40 ppm) giving a typical 1D NMR spectrum. The on- and off-resonance spectra are recorded under the same conditions with the exception of the selective RF pulse in different positions. The difference spectrum only shows resonances of (ligand) molecules which have

bound to the saturated protein and received saturation via spin diffusion from the intermolecular NOE. When the saturated ligand dissociates into solution it is detected (Figure 2.22). So, if no binding events occur, the difference spectrum will show no ligand signals. If binding affinity is too high (i.e. very low K_D), and the ligand does not dissociate on the NMR timescale (ms) then there will be no observable STD effect.

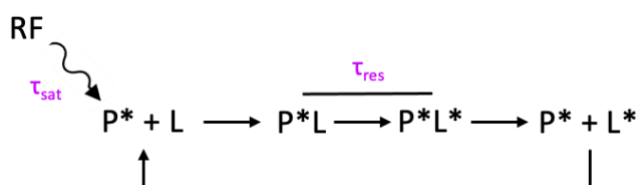


Figure 2.22 Saturation transfer difference (NMR) schematic. The protein (P) is saturated using a radiofrequency pulse. If a ligand (L) molecule binds to the saturated protein (P^*), it will receive saturation via the intermolecular NOE. The saturated ligand (L^*) will dissociate into solution and be detected in the ^1H NMR spectrum. The time for saturation and residence time of the ligand are shown as τ_{sat} and τ_{res} respectively.

The main disadvantage of STD NMR is the lack of discrimination between specific and non-specific ligand binding.¹⁷² The strength of the STD NMR signal depends on the concentration of ligand and the efficiency of saturation transfer, as well as the irradiation and saturation times.^{172,179} Ligand protons which are closer to the binding site of the protein will receive more efficient saturation and therefore have larger STD signals. This can be used for ligand binding epitope mapping (Figure 2.23). Differential epitope mapping (DEEP) STD NMR is a multi-frequency approach which has been developed to distinguish between non-specific ligand binding and the interactions in the binding pocket to overcome this issue.¹⁸¹

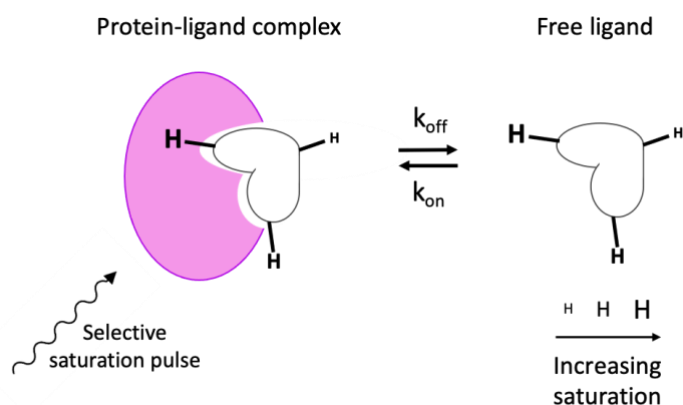


Figure 2.23 Ligand epitope mapping. Ligand resonances which are closer to the binding site of the saturated protein (pink) will receive more efficient saturation, as represented by the size of the H atoms. This will be observed in the NMR spectrum of the ligand when it dissociates into solution.

If a K_D value is known for a system using other techniques, the value obtained from the STD-NMR experiment can be compared. This has been done for Glt_{ph} , a protein with high sequence similarity to the main protein of interest in this thesis, Glt_{Tk} . The K_D value determined for glutamate binding to Glt_{ph} using STD NMR was comparable to values obtained using other methods (see Appendix A1).^{111,112,124,126,128,129,133,182} The analysis and interpretation of the STD NMR titration experiments will be discussed in detail in Chapter 6 .

3 Investigating the binding induced conformational changes of Glt_{Tk} using continuous wave EPR spectroscopy

The vast majority of published data on the glutamate transporters is based on a closely related archaeal homologue, Glt_{Ph}. The first crystal structure of Glt_{Ph} was published in 2004,¹⁰⁸ compared to Glt_{Tk} almost 10 years later in 2013.¹⁰⁹ Previous studies on Glt_{Ph} include the use of electron paramagnetic resonance (EPR) spectroscopy to investigate the conformational changes and associated mechanism using both continuous wave (cw) and pulsed EPR techniques. However, the results of these studies are mixed in terms of preferred conformations in given binding states.^{134,135,142} Currently there is no published data for EPR spectroscopy of Glt_{Tk}, which is a promising additional model system to Glt_{Ph} for studying the glutamate transporters. Overall, the two systems have similar global structures but there are subtle differences in binding sites for sodium and aspartate.¹⁰⁹ This chapter will discuss the production and purification of Glt_{Tk} in different protein environments; followed by spin labelling and investigation using cw-EPR spectroscopy.

3.1 Rationale for the choice of labelling sites

One fundamental requirement for EPR spectroscopy is the presence of a paramagnetic centre. Like most membrane transporter complexes, Glt_{Tk} does not contain an unpaired electron and therefore required the attachment of an exogenous spin label such as (1-Oxyl-2,2,5,5-tetramethyl-Δ3-pyrroline-3-methyl) Methanethiosulfonate (MTSL) which forms disulfide bridges with cysteine residues. Wild type (WT) Glt_{Tk} does not contain any native cysteine residues, therefore these were introduced using mutagenesis for site-directed spin-labelling (SDSL). Due to Glt_{Tk} being a trimeric protein, each of these variants were created to have a single cysteine inserted per protomer. In total, six Glt_{Tk} cysteine variants were studied: S281C, D298C, I312C, M314C, H334C and A367C (shown with the membrane topology in Figure 3.1). Each of these were designed for different biophysical studies at the University of Groningen, where their binding affinity for L-aspartate was also assessed using isothermal titration calorimetry (ITC) and transport using uptake

assays (Appendix A2). The suitability of each for EPR spectroscopy was then investigated using *in silico* analysis before the corresponding variant plasmids were acquired.

As discussed in Section 1.5.1, Glt_{TK} is composed of 8 α -helical transmembrane segments (TM) and two helical hairpins (HP1 and HP2) which are separated into the scaffold and transport domain. Each of the 6 cysteine variants are situated on mobile parts of the protein (transport domain) allowing the conformational changes and movement of the structure to be studied when binding Na⁺ and substrate molecules (Figure 3.2). S281C is situated on the topological domain between HP1a and HP1b which has been proposed to work as a gate to occlude the substrate binding site.¹⁰⁹ Within HP1 is a triple serine motif including S281 which is highly conserved in both archaeal and mammalian glutamate transporters.^{102,116} HP1 is largely intracellular, but the triple serine motif specifically is alternately accessible from either side of the membrane and is involved in substrate binding and dispatch of sodium ions to their binding sites making this an interesting variant to study.^{131,132} Therefore studying S281 may further elucidate Glt_{TK} gate movements upon Na⁺, substrate and inhibitor binding.

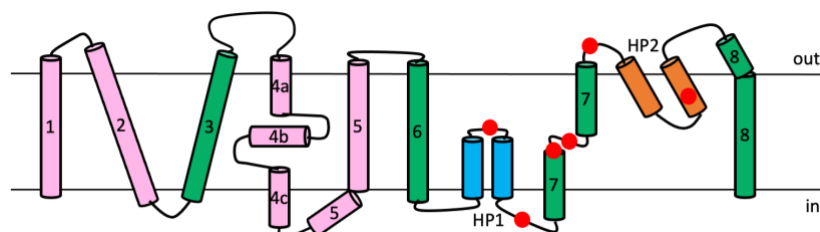


Figure 3.1 Glt_{TK} membrane topology. It is composed of a transport domain (green) and scaffold domain (pink), with HP1 (blue) and HP2 (orange) highlighted. Each of the variants are displayed as red dots, in numerical order from left to right (S281C, D298C, I312C, M314C, H334C, A367C).

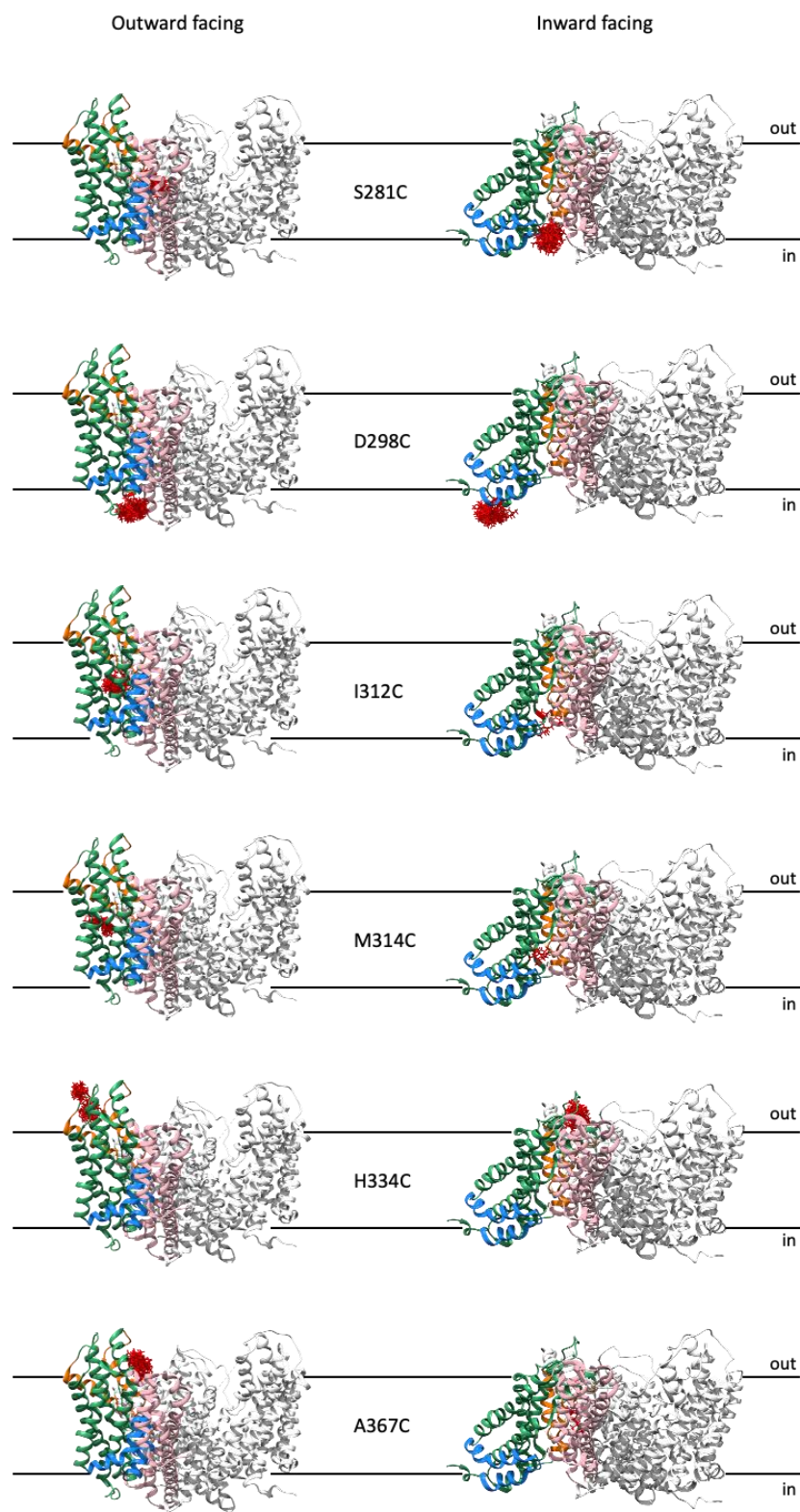


Figure 3.2 MMM results for Glt_{Tk} variants. Protomers are shown in an outward open (left, PDB 4KY0) and 2 inward open with 1 intermediate outward occluded (right, PDB 6XWR) with MTSL rotamers shown as red clusters. The transport domain (green) and scaffold domain (pink) are shown, with HP1 (blue) and HP2 (orange) highlighted.

The highly conserved NMDGT motif (on TM7) is part of the substrate binding pocket, where M314 is a member of the motif itself and I312 is a neighbouring residue. Neighbouring residue, N313 is involved in binding of both Na 1 and Na 3 and is proposed to be crucial for stabilisation of the motif. M314 undergoes relocation from being exposed to the lipid bilayer in an apo state, to being involved in coordination of Na 2 in substrate bound Glt_{Tk}. It is important to remember the glutamate transporters are proposed to exhibit cooperativity i.e. after binding of the first two sodium ions (Na 1 and Na 3) the protein is thought to undergo conformational changes, particularly the NMDGT motif, to optimise the positions of residues for subsequent binding of the substrate followed by Na 2.¹¹⁶ Studying I312 and M314 could inform on changes in the local environment of these residues, which are in close proximity of substrate and Na⁺ binding sites. M314C was specifically designed to investigate the role of this residue in substrate: ion stoichiometry- with previous studies of this variant in Glt_{ph} showing diminished cooperativity.¹⁴⁰ This study found that although cooperativity is impaired, and therefore turnover rate is affected, the stoichiometry does not change for the transport cycle.

Of the 6 Glt_{Tk} variants, D298C has the cysteine most exposed to the intracellular environment (Figure 3.1). This residue was used to gain information about the larger conformational changes of the protein on the intracellular side of the membrane. It is in close proximity to the NMDGT motif, located in topological domain between HP1b and TM7. This variant, along with A367C, has been successfully used for making synthetic nanobodies (sybodies) to control the orientation of Glt_{Tk} in proteoliposomes. Sybodies inactivate the right-side out orientation of Glt_{Tk} in the bilayer, allowing kinetic analysis of inside-out orientation.¹⁴¹ A367 is located on HP2 which is proposed to act as a gate to both the intracellular and extracellular environment, so has a crucial role in transport across the membrane.¹¹⁶ Therefore, A367C had the potential to allow elucidation of gate movement as part of the mechanism using EPR spectroscopy.

On the extracellular side of the membrane lies the topological domain between TM7 and HP2, where H334 is located. This residue differs between Glt_{Tk}, Glt_{ph} and EAAT3

(Appendix A3).¹¹⁶ The position of H334 shifts quite significantly during the transport cycle, and when combined with D298 on the intracellular side, gives an overall view of the larger scale movements on both sides of the membrane. This includes changes in different binding states on addition of Na⁺, substrate and inhibitor.

3.2 *In silico* molecular modelling of Glt_{TK}

To assess the suitability of cysteine variants for spin-labelling and EPR spectroscopy, a computational modelling programme called multiscale modelling of macromolecules (MMM) was used.¹⁸³ This software is an open-sourced MATLAB toolbox which uses protein data bank (PDB) structures to *in silico* attach spin label (MTSL) to selected residues. MMM enables users to determine how successful a variant may be for SDSL and EPR spectroscopy before being created in the laboratory. This approach can be used to design a series of cysteine variants in selected areas of interest for the protein i.e. on scaffold domains to show the fixed position of these residues during the transport cycle, or on highly mobile parts of the protein to exhibit their large scale movement during ligand binding. However, it should be noted that there are limitations to this approach due to the use of rigid crystal and cryo-electron microscopy (cryo-EM) structures which do not account for protein movement in solution. For this reason, not all conformations of a protein may be accessible using these structural determination methods.

Each of the six Glt_{TK} cysteine variants were assessed using MMM for a variety of PDB structures representing the different binding states. These included apo protein, with Na⁺, substrate bound (Na⁺ & L-aspartate) and inhibitor bound (Na⁺ & DL-*threo*-β-Benzyloxyaspartic acid (TBOA)). TBOA is a non-transported, competitive inhibitor of Glt_{TK} which binds to the substrate binding-site and prevents closure of the extracellular gate, HP2.^{108,122,184} The two main outputs from MMM were the populations of each rotamer position and their spatial distribution (Table 3.1). These can be represented visually as shown in Appendix A4. The population of rotamers represented the number of positions MTSL could sample at a given position, which in turn reflected how accessible that position was for successful attachment of MTSL.

Experimentally this is dependent on protein conformation at the time of spin-labelling.

One of the main applications of biological EPR spectroscopy is to follow conformational changes of proteins (via SDSL) using distance determination methods. Pulsed EPR techniques are used to measure distances between 2–8 nm, therefore acting as the target range for MMM distance outputs. Using PDB structures in various binding states allowed an understanding of how the distances between pairs of spin-labels may change during the transport cycle and informed on whether observing such changes was feasible experimentally. Due to the trimeric nature of Glt_{Tk}, for every cysteine variant there were three rotamer populations (A, B and C) and three distance distributions (AB, AC and BC). In many of the PDB structures the three protomers exhibited heterogeneity so the data for each of the protomers within a single structure varied. The conformation of each protomer in the PDB structure used is included in Table 3.1. After attachment of MTSL to the selected positions, the results were exported and used for interactive visualisation in Chimera. Here the number of rotamers were visually represented as clusters of MTSL residues attached to the PDB structure (Figure 3.1).

Generally, all six cysteine variants had a good number of rotamers in an apo state. For M314C, this declined significantly in the other three binding states where two of the three protomers were calculated to have one rotamer and the remaining protomers had <5 rotamers. This indicated M314C is an inaccessible residue and may be the most difficult to spin label. In an apo state, proteins are thought to have conformational fluctuations which would help accessibility for spin labels to bind. Therefore, M314C was tested experimentally despite these results as the plasmid was already created. In the Na⁺-inhibitor bound cryo-EM structure analysed, two of the three protomers have DL-TBOA bound in outward open states, with the third Na⁺ bound in an inward open conformation. An interesting observation in the MMM results for the number of rotamers was for A367C in an Na⁺-inhibitor bound state. For all other outward facing conformations of the variant, there were a large number of rotamers predicted (>50) but when DL-TBOA bound this decreased significantly to 1,

showing spin label movement and flexibility may be restricted by the benzyl ring which cannot fit in the substrate binding site (refer to Figure 1.14 for DL-TBOA structure).

Due to all six Glt_{TK} cysteine variants being part of the transport domain, they all had predicted changes in distance distributions to varying extents (Table 3.1). S281C was predicted to change from 2.4 nm in an apo state (outward facing conformation), to 4 nm upon binding Na⁺ and forming an inward-open conformation. These changes were unsurprising due to the residue being on the proposed intracellular gate which opens and closes to control access to the binding sites. Similarly, A367C which is part of HP2 (the extracellular gate) had predicted distance distributions which ranged between 2.5 nm and 5 nm for the various binding states. This contrasted to I312C which was proposed to undergo much more subtle changes in inter-spin distances with all distances staying between 4–5 nm for the transport cycle. The presence of shorter distances may be evident in the raw experimental time trace of double electron-electron resonance (DEER) experiments as higher frequency oscillations. However, as we approach longer the distances at the upper limit of pulsed EPR, experimental determination becomes challenging due to potentially unmeasurable oscillations. This was the case for H334C, with predicted distances as long as 7.45 nm which would be hard to observe experimentally. There are also the added difficulties associated with membrane proteins such as low labelling efficiency, low protein concentration and addition of cryoprotectants.

State	Conformation(s)	Variant	Rotamers	Distances (nm)
Apo ^I	Outward facing	S281C	35 / 39 / 34	2.48 / 2.41 / 2.39
		D298C	38 / 37 / 36	3.77 / 3.64 / 3.70
		I312C	35 / 23 / 24	4.52 / 4.77 / 4.76
		M314C	22 / 12 / 12	5.54 / 5.36 / 5.47
		H334C	18 / 21 / 16	6.68 / 6.96 / 6.84
		A367C	60 / 65 / 50	3.73 / 3.69 / 3.81
Na ⁺ only ^{II}	Intermediate-outward occluded apo / inward open Na ⁺ / inward open Na ⁺	S281C	84 / 1 / 73	4.28 / 4.01 / 4.02
		D298C	80 / 51 / 97	5.53 / 6.84 / 6.00
		I312C	2 / 16 / 2	4.41 / 4.01 / 4.46
		M314C	5 / 1 / 1	6.09 / 5.03 / 5.15
		H334C	24 / 11 / 30	4.96 / 3.63 / 4.25
		A367C	1 / 49 / 1	4.50 / 4.28 / 4.16
Substrate bound ^{III}	Outward-occluded Asp	S281C	1 / 3 / 4	2.36 / 2.45 / 2.97
		D298C	29 / 39 / 41	3.63 / 3.61 / 3.74
		I312C	15 / 8 / 15	4.69 / 4.74 / 4.94
		M314C	- / 1 / 1	- / - / 4.22
		H334C	45 / 40 / 46	7.42 / 7.44 / 7.45
		A367C	50 / 61 / 56	3.81 / 3.61 / 3.50
Substrate bound ^{IV}	Intermediate-outward occluded Asp	S281C	1 / 1 / 1	2.94 / 2.97 / 2.91
		D298C	51 / 61 / 66	4.39 / 4.41 / 4.40
		I312C	22 / 19 / 17	4.04 / 4.12 / 4.12
		M314C	1 / 1 / 2	3.27 / 3.39 / 3.14
		H334C	36 / 30 / 38	5.72 / 5.93 / 5.69
		A367C	71 / 75 / 72	2.73 / 2.59 / 2.69
Inhibitor bound ^V	Inward open Na ⁺ / outward open TBOA / outward open TBOA	S281C	79 / 37 / 32	4.34 / 4.32 / 2.70
		D298C	70 / 43 / 60	5.24 / 5.83 / 3.75
		I312C	2 / 9 / 11	4.55 / 4.09 / 4.22
		M314C	4 / 1 / 1	5.03 / 4.28 / 4.83
		H334C	26 / 104 / 62	6.08 / 5.24 / 7.11
		A367C	1 / 1 / 2	5.00 / 4.08 / 4.63

Table 3.1 Results of MMM analysis using structural models of Glt_{TK} spin labelled with MTSL. All six variants were analysed giving predictions of the number of rotamers on each protomer (A/B/C) and the distance distributions between pairs of residues (AB, AC, BC). A range of binding states and conformations were studied to see how the selected position moves during the transport cycle where substrate bound refers to sodium and L-aspartate, and inhibitor is with sodium and DL-TBOA. ^IPDB 4KY0, ^{II}PDB 6XWR, ^{III}PDB 5E9S, ^{IV}PDB 6XWQ, ^VPDB 6XWN.

3.3 Glt_{Tk} protein production, purification, and spin-labelling

All protein production and purification was carried out in-house, and the protocols to do so were optimised. For the purification of Glt_{Tk} A367C a comparison between TALON Metal Affinity Resin (Cobalt) and Ni Sepharose 6 Fast Flow resin was made. These both use the principles of immobilised metal affinity chromatography (IMAC), but cobalt resin offers better specificity and protein purity. This was confirmed by Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE, Figure 3.3) where significantly less contaminants were present in the sample, so TALON resin was used for all purifications. The alternative Ni Sepharose resin was used for SMALP

purifications of Glt_{Tk} S281C and A367C as this had been used for SMALP preparations previously at the industrial partner, Peak Proteins.

For SDSL, purified protein was incubated with MTSL overnight before undergoing wash steps to remove the free label. Two approaches to this were tested: spin-labelling during the purification process (whilst the protein was still bound to the resin), and spin-labelling after the protein was purified. Due to protein production and purification being carried out in-house both options were available. One of the main differences between these two methods was incubation with dithioreitol (DTT) when labelling after purification. This ensured the cysteine residues were reduced. DTT was then washed off using Zeba™ Spin desalting columns before the incubation step with MTSL. Due to incompatibility of TALON resin with DTT, and the alternatives of dithioerythritol (DTE) and TRIS (2-carboxyethyl) phosphine (TCEP), this reducing step was not used when spin-labelling on the column. Instead, the MTSL incubation step was inserted in the middle of wash 1. Both protocols involved incubation with MTSL overnight, at 4°C, with excess spin-label being removed the next day. For

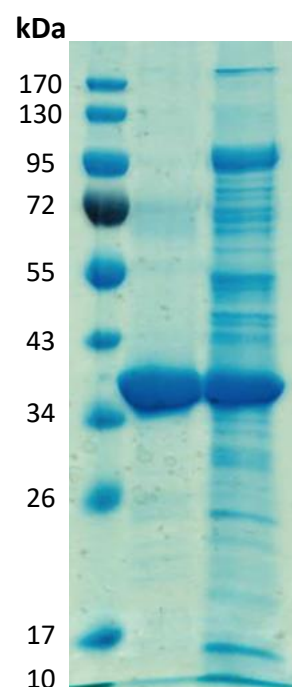


Figure 3.3 SDS PAGE of Glt_{Tk} A367C purified using cobalt TALON Metal Affinity Resin (centre) and Ni Sepharose 6 Fast Flow resin (right).

labelling on the column this involved continuation of the purification protocol (i.e. half of wash 1, wash 2 and protein elution). For SDSL of purified protein, Zeba™ Spin desalting columns were used again to remove excess MTSL. Similar protein yields and comparable spin-labelling efficiencies were obtained for both methods (0.31 mg/L culture with 90% labelling efficiency for purified protein vs 0.38 mg/L culture and 100% for labelling on the column). Figure 3.4 shows the continuous-wave (cw) EPR spectra recorded for this comparison. It was evident for the sample labelled on the column there was slightly more free-MTSL however this was negligible. Therefore, labelling during purification was employed in all SDSL to reduce number of steps and handling of the protein samples which could result in sample loss and degradation.

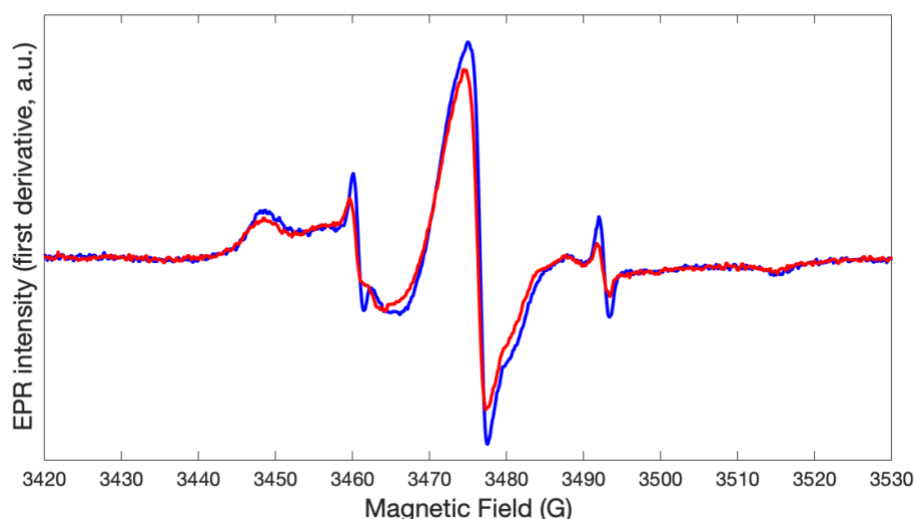


Figure 3.4 X-band RT cw-EPR spectra of apo GlT_{TK} S281C following SDSL during purification (blue) and after purification (black). Experimental parameters: microwave frequency = 9.758 GHz, centre field = 3478 G, sweep width = 120 G, modulation frequency = 100 kHz, modulation amplitude = 1G, microwave power = 0.1997 mW, number averages = 64.

All six GlT_{TK} variants were purified in detergent and spin labelled on the column. Spin labelling was assessed using cw-EPR where each protein sample was recorded in an apo state first. To calculate spin-labelling efficiency, a series of 2,2,6,6-tetramethylpiperidiny-1-oxyl radical (TEMPO) standards were recorded under the same conditions as the protein sample(s) and the double integration value for each was used to plot a calibration curve (e.g. Figure 3.5). The concentration of spin-label in the protein sample was then determined using the line of best fit from

the calibration curve and the double integral values of the protein cw-EPR spectrum. The spin-labelling efficiency was then calculated by dividing the concentration of MTSL by the protein concentration. The protein yield and respective labelling efficiency for each Glt_{Tk} variant can be seen in Table 3.2. The efficiency of Glt_{Tk} H334C (>100%) may be a result of the presence of free-MTSL because this method takes the area under the cw-EPR spectrum and did not distinguish between free and bound MTSL.

	S281C	D298C	I312C	M314C	H334C	A367C
Yield (mg/L)	0.41	0.41	0.58	0.18	0.42	0.29
Labelling efficiency (%)	65	87	60	44	123	97

Table 3.2 Glt_{Tk} protein yield for each variant and the calculated spin labelling efficiency ($\epsilon = 37360 \text{ M}^{-1} \text{ cm}^{-1}$, $M_W = 46642.64 \text{ g/mol}$)

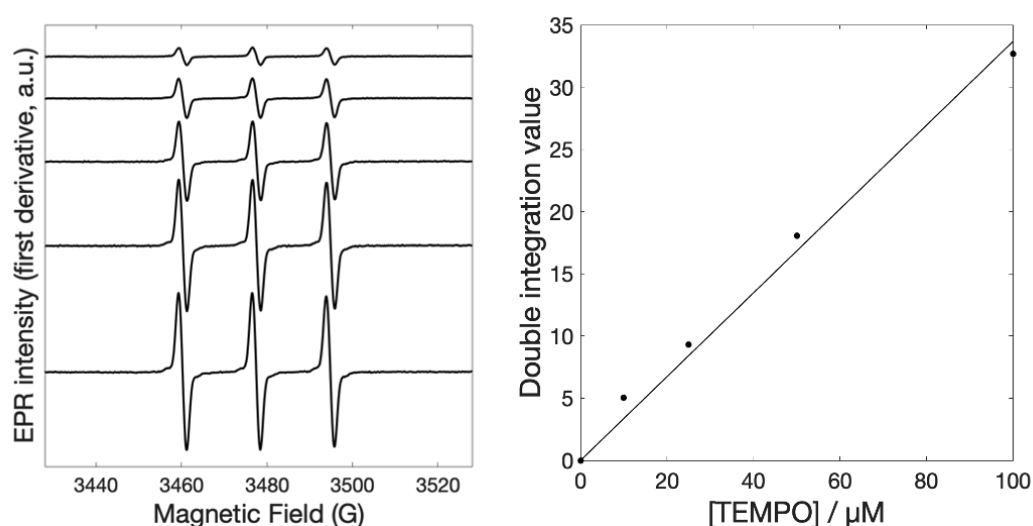


Figure 3.5 X-band RT cw-EPR spectra of TEMPO (left, top to bottom: 10 μM , 25 μM , 50 μM , 75 μM , 100 μM). The double integration values for each were used to create a calibration curve (right). Experimental parameters: microwave frequency = 9.758 GHz, centre field = 3478 G, sweep width = 120 G, modulation frequency = 100 kHz, modulation amplitude = 1G, microwave power = 0.1982 mW, number averages = 4–16.

The cw-EPR spectra of all six spin labelled Glt_{Tk} variants in an apo state were significantly different to TEMPO which represented free spin-label in solution (Figure 3.6). This confirmed successful binding of MTSL to the cysteine residues of the protein. Each spectrum was also different to each other and contained varying levels

of free-MTSL present. The different line shapes reflected the distinctive environment of each spin-labelled position, which is discussed in more detail in the rest of the chapter.

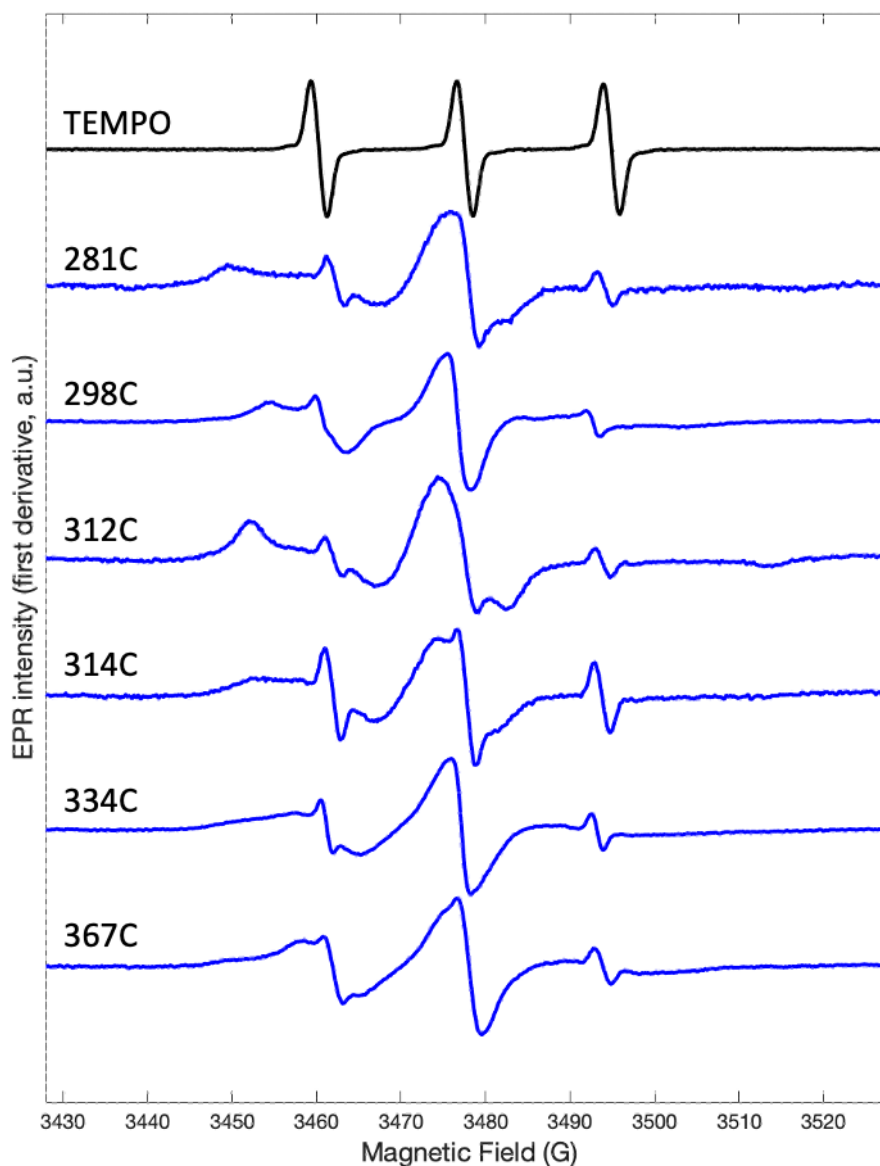


Figure 3.6 X-band RT cw-EPR spectra of all six Glt_{Tk} variants in an apo state (blue). TEMPO (black) is shown as reference of unbound, free spin-label. Experimental parameters: microwave frequency = 9.758 GHz, field modulation frequency = 100 kHz, modulation amplitude = 1G, microwave power = 0.1997 mW, number averages = 64, time constant = 20.48 ms, conversion time = 20.48ms.

This chapter will now present and discuss the results of cw-EPR spectroscopic studies for each of the 6 Glt_{Tk} variants separately. Functional studies were conducted to

follow the transport cycle of Glt_{Tk} through addition of substrate or inhibitor molecules to apo-protein. This included the investigation of different protein environments: detergent micelles, proteoliposomes, styrene maleic acid lipid particles (SMALPs) and nanodiscs.

3.4 Studying the conformational changes of Glt_{Tk} S281C using cw-EPR spectroscopy

3.4.1 Functional studies of Glt_{Tk} S281C in detergent micelles using cw-EPR

In order to investigate the conformational changes and associated dynamics during the transport mechanism, variant Glt_{Tk} S281C was selected due to the close proximity to the substrate binding site. Studies using continuous wave (cw) EPR spectroscopy were conducted at ambient temperature. Figure 3.7 shows the spectra obtained utilising substrate (L-aspartate), inhibitor (DL-TBOA) and co-transported Na⁺ ions to trap Glt_{Tk} in proposed intermediates of the transport cycle.

Subtle differences in line shape observed between the four intermediates of the transport cycle in Glt_{Tk} S281C are indicative of the protein undergoing conformational change as proposed by *in silico* analysis of Glt_{Tk} in different binding states (Section 3.2). As shown in Section 2.1.3, a broader overall spectral width EPR signal is indicative of a longer rotational correlation time (τ_c) and therefore a more restricted local environment of the spin-label. A small residual contribution from unbound, free MTSL can be seen in the cw-EPR spectra as small sharp peaks superimposed on the broad spectral line shapes of the spin-labelled protein. There are clear differences in two main regions of the EPR spectra, highlighted in Figure 3.7. These could represent two distinct conformations of Glt_{Tk} in equilibrium whose intensity varies as the protein binds substrate molecules and Na⁺ ions. Published structures of Glt_{Tk} exhibit heterogeneity where the three protomers are in two different conformations (except when substrate bound), with different rotamer distribution predictions for each based on *in silico* analysis.¹²² The four binding states differ from each other with Na⁺-only and Na⁺-inhibitor bound states being most similar, with a greater proportion

of a species with a fast rotational correlation time. Quantification of these effects will be discussed later in Section 3.4.6.

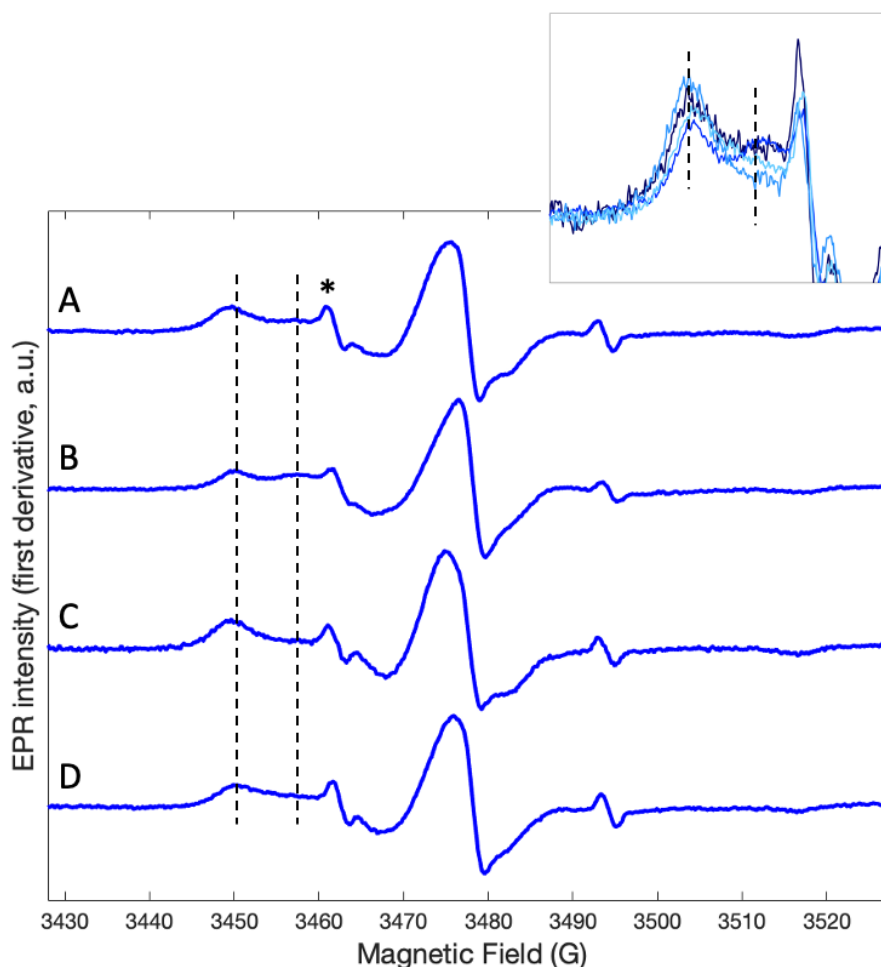


Figure 3.7 X-band RT cw-EPR spectra of Glt_{Tk} S281C in detergent micelles utilising substrate and co-transported Na⁺ to trap Glt_{Tk} in proposed intermediates of the transport cycle. A) apo, B) Na⁺ (50 mM), C) substrate bound (50 mM Na⁺ and 1 mM L-aspartate) and D) inhibitor bound (50 mM Na⁺ and 1 mM DL-TBOA). Black dashed lines indicate regions of the spectra where subtle changes are observed (see insert). Zoomed in area of the biggest shape changes in the binding conditions shown darkest to lightest for A) to D). Residual free-MTSL is present as three sharp peaks (*). Experimental parameters: microwave frequency = 9.758 GHz, field modulation frequency = 100 kHz, modulation amplitude = 1G, microwave power = 0.1997 mW, number averages = 64, time constant = 20.48 ms, conversion time = 20.48 ms.

A Na⁺ concentration of 50 mM for initial EPR experiments was selected based on a previous EPR study of Glt_{Ph}.¹³⁴ However, Glt_{Tk} structural data used a Na⁺ concentration of 300 mM and recent EPR studies of Glt_{Ph} used 200 mM NaCl.^{122,145,185} As a control, the cw-EPR studies were repeated using 300 mM Na⁺ to ensure there was no effect

on spectral line shapes. The resulting spectra were identical to those previously recorded using 50 mM Na⁺ with the only differences being the amount of free-MTSL present in the samples (Figure 3.8).

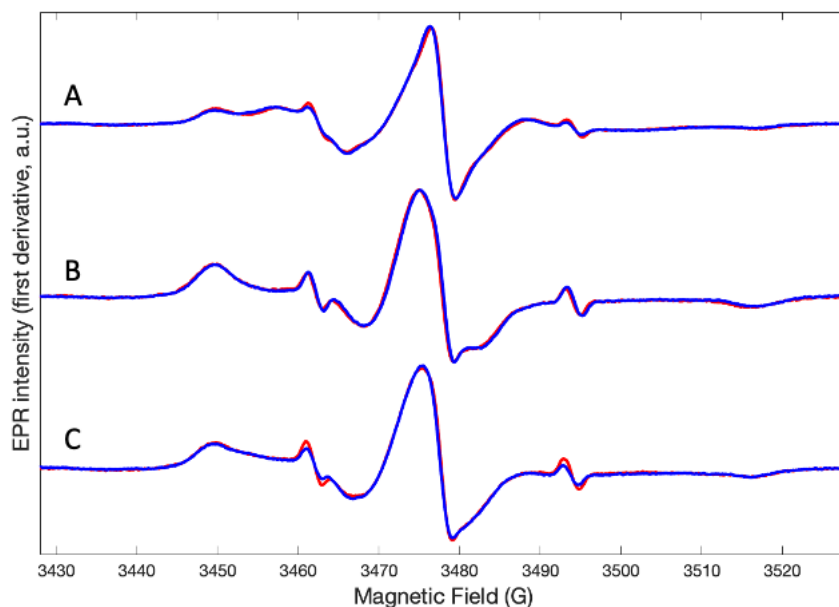


Figure 3.8 X-band RT cw-EPR spectra of Glt_{Tk} S281C in detergent micelles using Na⁺ concentrations of 50 mM (red) and 300 mM (blue). A) Na⁺, B) substrate bound (Na⁺ and 1 mM L-aspartate) and C) inhibitor bound (Na⁺ and 1 mM DL-TBOA). Experimental parameters: microwave frequency = 9.758 GHz, Field modulation frequency = 100 kHz, modulation amplitude = 1G, microwave power = 0.1997 mW, number averages = 64, time constant = 20.48 ms, conversion time = 20.48 ms.

3.4.2 Stability of spin labelled Glt_{Tk} S281C in detergent micelles

The small sharp peaks seen previously (Figure 3.6) are indicative of residual free-MTSL in the detergent micelle samples. This could be due to spin-label which did not bind to the cysteine residue and/or was not removed during the protein washing and concentration steps. Spin labelled Glt_{Tk} S281C (apo state) was studied over time, a cw-EPR spectrum was recorded regularly for a period of ~64 hours. The intensity of one of the free-label peaks (Figure 3.7, *) was plotted against time (Figure 3.9). Over time the free-MTSL signal increases. After ~64 hours this began to plateau at approximately 4 times the original MTSL signal intensity. However, even following 64 hours, the spin-labelled protein signal is still the most abundant species in the

overall cw-EPR spectrum, denoting that significant detachment of spin label does not occur over many hours. Often membrane proteins, particularly in detergent, are kept at 4°C so some sample degradation is expected as the experiment was performed at room temperature (RT). Changes in spectral line shape or signal intensity of the protein species could indicate protein degradation through a change in spin-label environment and would be a point of observation in the future. Factors such as the temperature of the sample and changes in volume should also be observed. Alternative techniques to monitor the sample stability could be used such as mass spectrometry and melting point measurements (comparing labelled and unlabelled protein to wild-type over time).

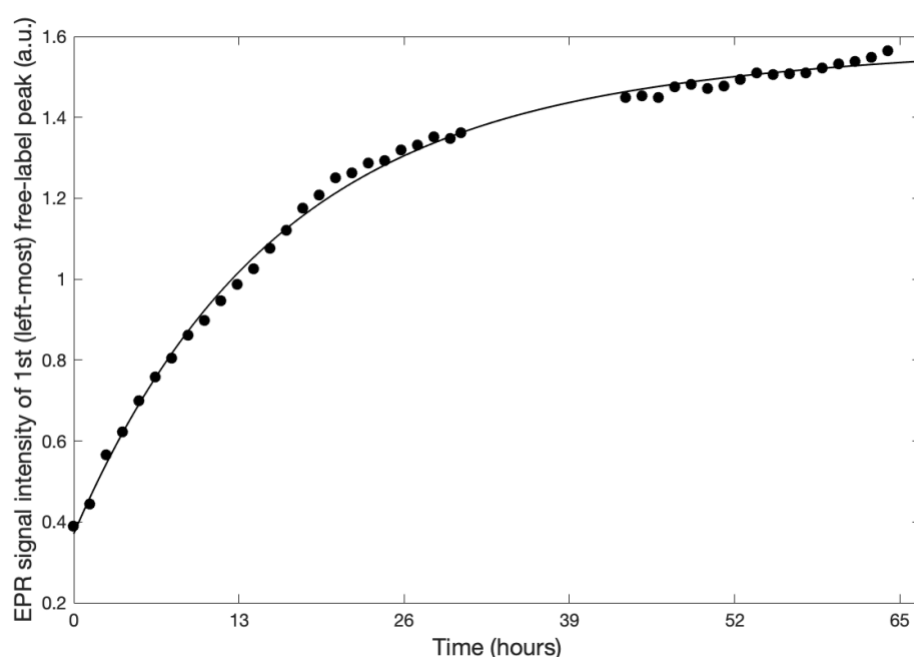


Figure 3.9 The evolution of the free-MTSL signal in apo Glt_{TK} S281C over time. This was analysed by plotting one of the peaks assigned to the free-MTSL peak in the X-band RT cw-EPR spectrum at timed intervals.

3.4.3 Spin labelled Glt_{TK} S281C studied in more biologically relevant environments

Biophysical characterisation of membrane proteins is commonly performed in detergent solubilised micelles for ease of handling and often to ensure high concentrations necessary for complex biophysical methods can be achieved.

However, they do not necessarily provide a good model for the proteins natural environment. In order to validate data acquired from samples in detergent micelles, cw-EPR experiments were repeated in a series of environments chosen to mimic the natural membrane: proteoliposomes, SMALPs and nanodiscs (Figure 1.2). Protocols for reconstitution into proteoliposomes and formation of Glt_{7k} SMALPs were optimised in-house and during visits to the industrial partner, Peak Proteins, respectively (see Section 8.1.4). The most challenging aspect for the formation of proteoliposomes is achieving the sample volume and concentration required for successful EPR studies. Nanodisc reconstitution was completed by collaborators at the University of Groningen using spin-labelled, detergent-purified Glt_{7k} protein produced in-house.

The process of proteoliposome reconstitution uses detergent solubilised protein which is embedded into a lipid bilayer of chosen composition to yield a membrane-like environment with defined internal and external environments.^{13,15} This contrasts to SMALPs which have been compared to a “cookie cutter” where proteins are extracted from their surrounding native membrane lipids meaning that the membrane protein has never been in contact with detergent molecules. Nanodiscs have been used in previously published structural studies of Glt_{7k} where the protein is encapsulated in a “disc-like” structure held together by scaffold proteins.¹²² For reconstitution into a nanodisc and proteoliposome environment, the membrane protein must first be extracted into detergent-solubilised micelles.^{13,17} Therefore, Glt_{7k} was spin-labelled during the detergent micelle purification steps for these two membrane mimetics. Due to the different extraction and purification procedures, Glt_{7k} S281C SMALPs were spin labelled after encapsulation.

Since each of these three more native environments may reveal subtle spectral differences, results for Glt_{7k} S281C are compared and contrasted. The overall shape of the cw-EPR spectra of apo Glt_{7k} S281C in each of these environments is similar to Glt_{7k} S281C in detergent micelles (Figure 3.10). The cw-EPR spectra for these three alternative environments in the four binding states are shown in Figure 3.15–Figure 3.17 including their respective spectral simulations. Most interestingly, in detergent

micelles and nanodiscs the spectra for all four intermediate states of the transport cycle were subtly different to each other, however for Glt_{TK} S281C in proteoliposomes the Na⁺-bound, and Na⁺-inhibitor bound states appeared identical, while the apo and Na⁺-substrate bound states were very similar to each other. For Glt_{TK} in SMALPs the spectral line shapes were similar in each of the binding states.

Spectra recorded in these three closer-to-native environments also exhibited less contribution from free-MTSL signal which may be due to the extra wash steps in the protein reconstitution processes. When comparing all environments for each respective intermediate in the transport cycle the main differences observed were in all cases the relative populations of the two distinct conformations of Glt_{TK} discussed previously. These results were quantified using spectral simulations discussed in Section 3.4.6.

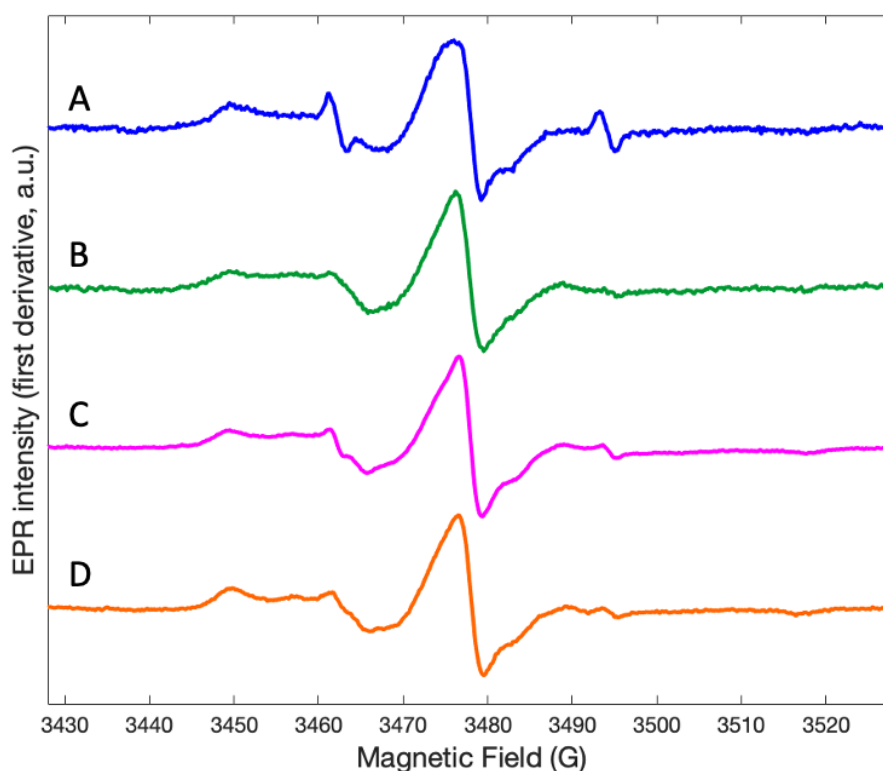


Figure 3.10 X-band RT cw-EPR spectra of apo Glt_{TK} S281C in A) detergent micelles (blue), B) proteoliposomes (green), C) SMALPs (pink) and D) nanodiscs (orange). This colour scheme will be used throughout the thesis. Experimental parameters: microwave frequency = 9.758 GHz, Field modulation frequency = 100 kHz, modulation amplitude = 1–2 G, microwave power = 0.1997 mW, number averages = 64–124, time constant = 20.48 ms, conversion time = 20.48 ms.

3.4.4 Studying immobilised spin-labelled Glt_{7k} S281C using cw-EPR spectroscopy at X-band (9.4 GHz)

To achieve a full understanding of the spectral contributions observed, knowledge of a whole range of spin-Hamiltonian parameters including g-values, hyperfine constants (A-values), rotational correlation time etc. is required which in turn allows us to investigate the mechanism of transport. Some of these can be determined using EPR at cryogenic temperatures which immobilises the spin-labelled protein. Spin labelled Glt_{7k} S281C was studied using cw-EPR spectroscopy at cryogenic temperatures. At X-band, 50K is usually sufficient to observe the hyperfine constant along the z-axis (A_{zz}).

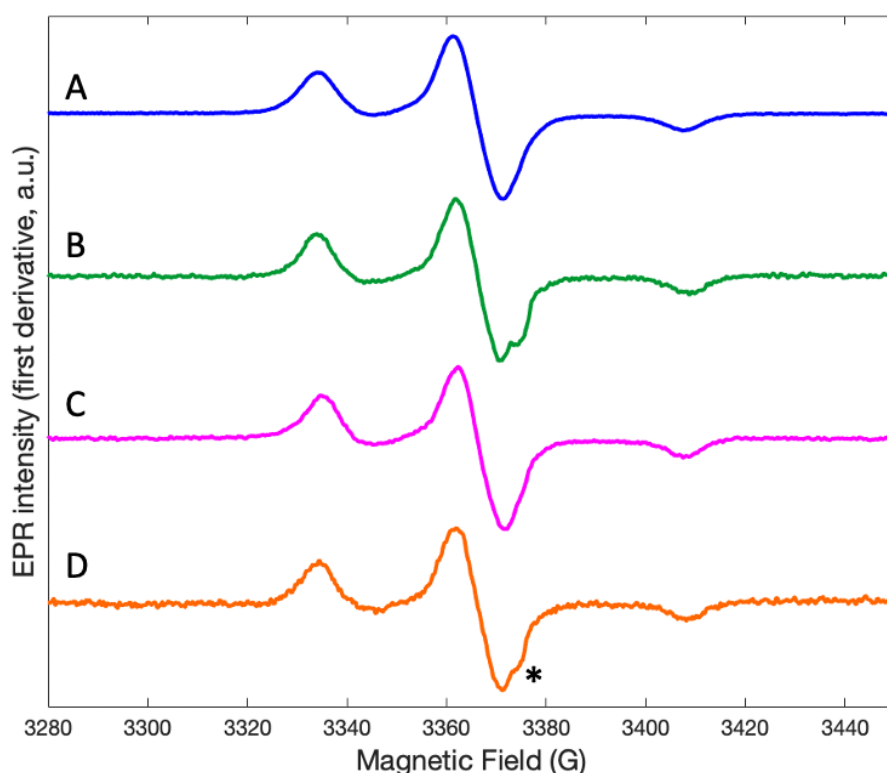


Figure 3.11 X-band LT cw-EPR spectra (at 50 K) of apo Glt_{7k} S281C in A) detergent micelles (blue), B) proteoliposomes (green), C) SMALPs (pink) and D) nanodiscs (orange). A shoulder of the central peak thought to be a result of trapped oxygen is highlighted (*). Experimental parameters: microwave frequency = 9.439 GHz, centre, field modulation frequency = 100 kHz, modulation amplitude = 2 G, microwave power = 0.01254–0.00315 mW, number averages = 9–49, time constant = 40.96 ms, conversion time = 40.96 ms.

Figure 3.11 shows the LT cw-EPR spectra of apo Glt_{7k} S281C in detergent micelles, proteoliposomes, SMALPs and nanodiscs. All cw-EPR spectra were the same for each

environment under different binding conditions (Appendix A5) indicating the changes in line shape of the RT cw-EPR spectra were a result of changes in the mobility of the spin label. The central peak had a shoulder in some LT cw-EPR spectra, particularly at lower protein concentrations (highlighted as an * in Figure 3.11). This peak was believed to be the result of trapped oxygen in the sample tube and not a from the protein itself. This appeared most frequently in proteoliposome samples which often shifted up the EPR tubes due to the expansion of gas trapped at the bottom of the sample.

3.4.5 Studying immobilised spin-labelled Glt_{TK} S281C using cw-EPR spectroscopy at Q-band (34 GHz)

Moving to higher frequency improves spectral resolution, and therefore allows determination of more EPR parameters such as hyperfine constants (A-values) and g-values along the cartesian axis. However, increasing frequency means more difficulty acquiring the spectra, with LT X-band being harder than RT X-band and in turn LT Q-band being the most complex. LT Q-band spectra were recorded for Glt_{TK} S281C in detergent micelles in an apo, Na⁺-bound, Na⁺-substrate bound, and Na⁺-inhibitor bound states. The overall spectral line shapes were similar, confirming the changes in line-shape observed in the RT spectra were a result of varying τ_{cs} i.e. change in the spin label mobility. These data are consistent with the LT X-band spectra (Figure 3.12).

3.4.6 Spectral simulations of Glt_{TK} S281C using EasySpin

To extract numerical parameters from cw-EPR spectra, a software package called EasySpin was employed. This was an iterative process utilising all the spectra obtained at different microwave frequencies and temperatures. The Q-band cw-EPR spectra obtained for Glt_{TK} S281C in detergent micelles were used to extract g and A values which were kept fixed in all iterative simulations of corresponding LT and RT X-band spectra. However, when Q-band cw-EPR is not available, the g_{zz} and A_{zz} can be extracted from LT X-band spectra - where $2A_{zz}$ is the distance between the outermost

peaks. The purpose of the simulations was to extract parameters which could be used to gain information about the local environment of the spin label, with the most informative parameter being τ_c from the RT spectra. Each simulation used the experimental microwave frequency and magnetic field (centre and sweep width) for the specific spectrum being fit.

Each time the spectrometer was reconfigured i.e. the cavity was changed, a standard sample (typically DPPH, or in the case of Q-band weak pitch) was measured and the resulting spectrum was simulated. The difference between the g-value determined from the recorded spectrum and the literature value of the standard provided the offset and therefore allowed correction for any simulated values. This was applied to all simulation results presented in this thesis to give the corrected values.

3.4.6.1 Simulating cw-EPR spectra of Glt_{rk} S281C in detergent micelles

For the first simulation of Glt_{rk} S281C apo in detergent micelles at LT Q-band, typical nitroxide radical values were used as a starting point. Once a good simulation was obtained the output values were assessed, and if required the variance and starting values of the simulation were altered until a final result was obtained. For subsequent Q-band spectra in the three binding states, the apo Q-band simulation results were used as starting values and allowed to vary to achieve the best result. Visually there was very little difference between the LT Q-band spectra for Glt_{rk} S281C in detergent micelles in all four binding states (Figure 3.12). The output results in Table 3.3 reflect this similarity, particularly in the g_{zz} and A_{zz} values where the similar values were found to simulate all spectra. When a sample is frozen the effect of τ_c is removed so the most informative value is the A_{zz} where a higher value (and lower g_{xx}) corresponds to a more polar environment. Therefore, the similar A_{zz} values for Glt_{rk} S281C in detergent micelles indicated the polarity of the spin label was not changing significantly.

	g_{zz}	g_{yy}	g_{xx}	A_{xx} (MHz)	A_{yy} (MHz)	A_{zz} (MHz)	Lwpp (mT)
Apo	2.0078	2.0054	2.0015	16.450	11.269	102.794	0.69
Na⁺	2.0078	2.0054	2.0015	17.457	11.472	102.712	0.69
Substrate	2.0078	2.0054	2.0016	15.784	10.336	102.792	0.69
Inhibitor	2.0078	2.0055	2.0016	15.364	12.139	102.797	0.65

Table 3.3 Best fit simulation values for LT Q-band cw-EPR spectra of Glt_{TK} S281C in detergent micelles. Results are shown for the four binding states with RMSD values of 0.4675 (apo), 0.4699 (Na⁺), 0.3696 (Na⁺ and L-aspartate), and 0.3142 (Na⁺ and DL-TBOA). The x, y, and z components of the g- and A-tensor and the linewidth (peak to peak, lwpp) are shown for each.

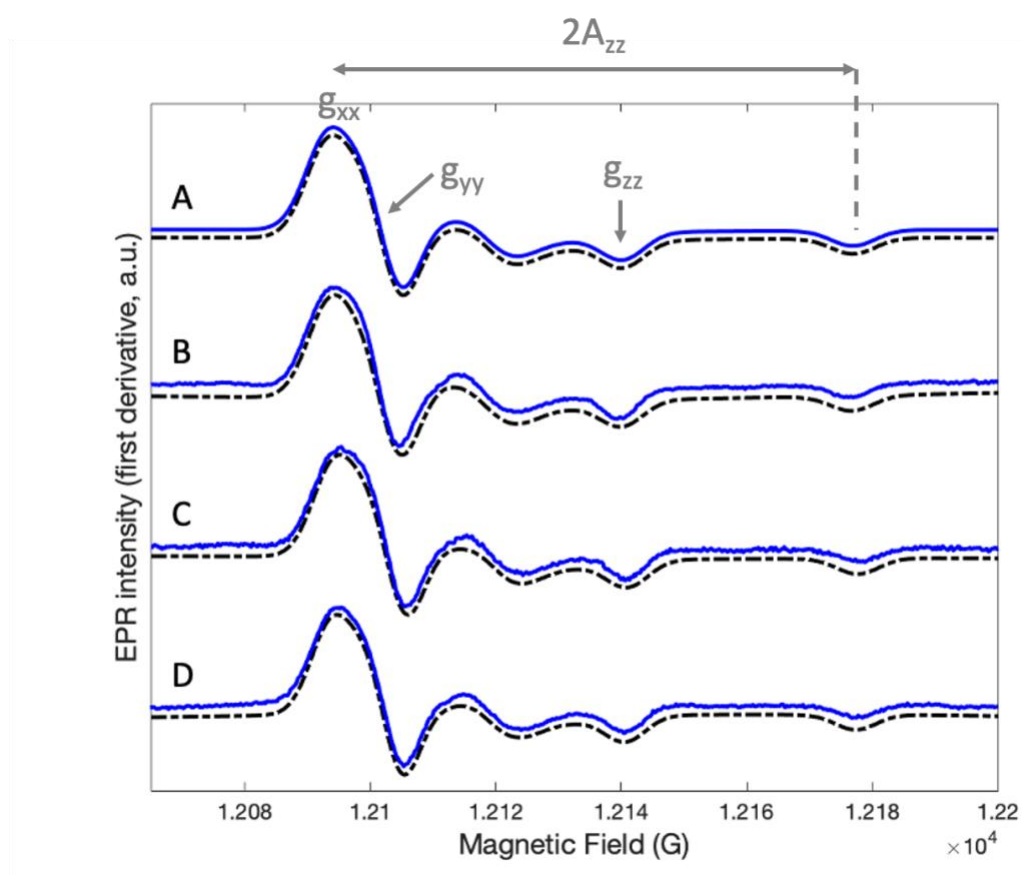


Figure 3.12 LT Q-band cw-EPR spectra for Glt_{TK} S281C in detergent micelles (blue) and their respective simulations using EasySpin (black dashed line) for the proposed intermediates of the transport cycle: A) apo, B) Na⁺ (50 mM), C) substrate bound (50 mM Na⁺ and 1 mM L-aspartate) and D) inhibitor bound (50 mM Na⁺ and 1 mM DL-TBOA)

The next stage of the simulation iteration was to apply these values obtained from the Q-band simulation to the LT X-band cw-EPR spectra, keeping the A and g values fixed and varying linewidth peak-to-peak (lwpp). These results are presented in Appendix A5.1, which correspond to those of LT Q-band.

If spectra at two different frequencies are available i.e. Q-band and X-band, it is possible to use a multifrequency fit within EasySpin which simultaneously fits both spectra together. This was used for Glt_{TK} S281C apo in detergent micelles where the experimental parameters for both experiments were included in the simulation script. The output when simulating both frequencies simultaneously has a less good fit than simulating them separately (Table 3.4 and Figure 3.13). The two different frequencies and experimental set-up had different offset values which are not accounted for in a multifrequency simulation script which may account for the less close fit. Therefore, this multifrequency fit method was not employed to simulate the rest of the spectra. Instead, they were simulated separately as part of the iterative process starting with LT Q-band (where available), followed by LT X-band and RT X-band.

	g_{zz}	g_{yy}	g_{xx}	A_{xx} (MHz)	A_{yy} (MHz)	A_{zz} (MHz)	Lwpp (mT)
Apo	2.0072	2.0050	2.0013	19.663	13.605	102.759	0.82

Table 3.4 Best fit simulation values using the multifrequency simulation function for LT Q-band and LT X-band cw-EPR spectra of apo Glt_{TK} S281C in detergent micelles (RMSD 0.0490).

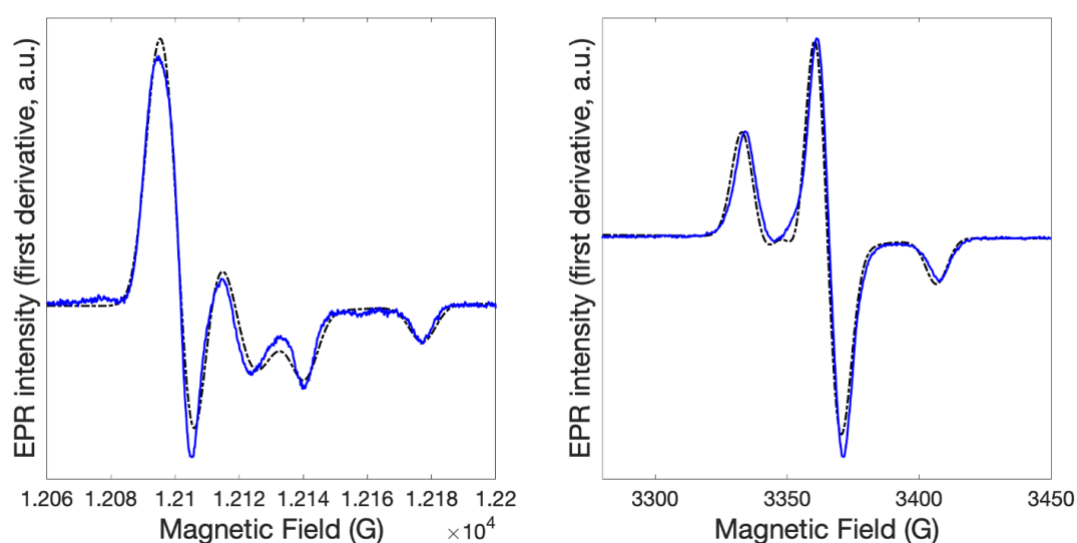


Figure 3.13 LT Q-band (left) and LT X-band (right) cw-EPR spectra for apo Glt_{TK} S281C in detergent micelles (blue) and their respective simulations using a multifrequency approach in EasySpin (black dashed line).

The more complicated the EPR spectrum, the harder it is to fit. This was the case for RT cw-EPR spectra where the protein is freely tumbling in solution, giving rise to additional parameters such as τ_c . There is also the additional complication of multiple protein conformations which no longer appear as the same line shape due to different spin label mobilities at RT. Different line shapes would be seen at LT if the protein conformations had different polarities represented by different A_{zz} 's. The shorter the τ_c , the more rotationally free the species is. With the opposite argument being true; the longer the τ_c , the more restricted the motion of the protein and therefore the broader the line shape. The τ_c is composed of the protein motion as a macromolecule in solution, the backbone motion of the protein where the spin-label is attached and the rotational motion of the spin-label side chain at the point of attachment. These all contribute to the different spectral line shapes which was the parameter of most interest.¹⁵⁵ Therefore different τ_c s have been associated with different conformational states of the protein.

To simulate the RT spectra, three species were used and therefore three sets of parameters. These represented two protein species (or conformations) and any residual free-MTSL present in the samples from spin-labelling. This was determined after attempting simulation with one protein species and free-MTSL which did not achieve a good fit. The g and A values extracted from the LT Q-band spectral simulations were fixed for the two protein species, with $lwpp$ and τ_c allowed to vary within the defined limits. Typical values for nitroxide spin label were used to simulate the residual MTSL signal, where the ratio in each of the four samples was varied to reflect the different amount of free MTSL present. The three species were also varied in their relative contributions to the spectral line shape. One protein species was assigned as 1, and the other two varied in their relative proportion to this - giving a ratio of the three species. The results of the fitted RT X-band spectra of Glt_{Tk} S281C in detergent micelles are shown in Table 3.5 and Figure 3.14 with the aim for elucidate the conformational changes during the transport cycle.

	g_{zz}	g_{yy}	g_{xx}	A_{xx} (MHz)	A_{yy} (MHz)	A_{zz} (MHz)	Lwpp (mT)	τ_c (ns)	Ratio
Apo	2.0078	2.0054	2.0015	16.450	11.269	102.794	0.16	6.5	1
	2.0078	2.0054	2.0015	16.450	11.269	102.794	0.21	37.9	0.32
	2.0077	2.0050	2.0021	8.611	14.551	109.113	0.10	0.6	0.05
Na⁺	2.0078	2.0054	2.0015	17.457	11.472	102.720	0.23	17.5	1
	2.0078	2.0054	2.0015	17.457	11.472	102.720	0.17	6.0	0.75
	2.0077	2.0050	2.0021	8.611	14.551	109.113	0.10	0.6	0.05
Substrate	2.0078	2.0054	2.0016	15.784	10.336	102.792	0.21	42.3	1
	2.0078	2.0054	2.0016	15.784	10.336	102.792	0.20	9.7	0.29
	2.0077	2.0050	2.0021	8.611	14.551	109.113	0.10	0.6	0.03
Inhibitor	2.0078	2.0055	2.0016	15.364	12.139	102.797	0.20	22.9	1
	2.0078	2.0055	2.0016	15.364	12.140	102.797	0.18	7.0	0.70
	2.0077	2.0050	2.0021	8.611	14.551	109.113	0.10	0.6	0.06

Table 3.5 Best fit simulation values for RT X-band cw-EPR spectra of Glu_{Tk} S281C in detergent micelles. Results are shown for the four binding states with RMSD values of 0.0306 (apo), 0.0427 (Na⁺), 0.0339 (Na⁺ and L-aspartate), and 0.0360 (Na⁺ and DL-TBOA). The x, y, and z components of the g- and A-tensor and the linewidth (peak to peak, lwpp) are shown for each.

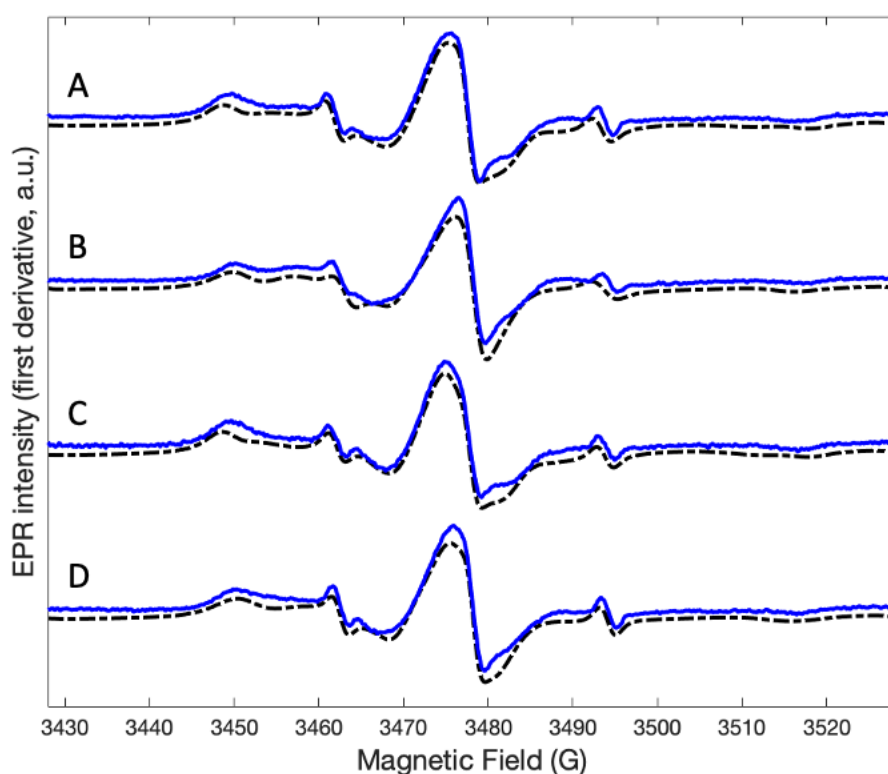


Figure 3.14 RT X-band cw-EPR spectra for Glt_{TK} S281C in detergent micelles (blue) and their respective simulations using EasySpin (black dashed line) for the proposed intermediates of the transport cycle: A) apo, B) Na⁺ (50 mM), C) substrate bound (50 mM Na⁺ and 1 mM L-aspartate) and D) inhibitor bound (50 mM Na⁺ and 1 mM DL-TBOA)

It is evident from the relative ratios of the three species that there is very little free MTSL present in each sample. Due to the fast τ_c the presence of free-MTSL may visually appear higher than the ~3% determined from simulation. Each Glt_{TK} S281C detergent micelle sample was simulated keeping the A and g-values extracted from LT Q-band fixed. These results confirm the observed line shape changes discussed previously in Section 3.4.1 are a result of τ_c . This means the rotational freedom of the spin-label changes upon addition of Na⁺ and substrate/inhibitor which is consistent with conformational changes in response to Na⁺ and substrate/inhibitor binding.

In apo Glt_{TK} S281C, the most abundant protein species has a much smaller τ_c (<10 ns) meaning the spin-label can rotate more freely. The opposite effect is observed for Na⁺, Na⁺-substrate and Na⁺-inhibitor bound Glt_{TK} S281C, where the most abundant species has the larger τ_c s. When comparing these results to *in silico* predictions

(MMM) based on crystal structures, the slower τ_c s may represent outward orientated states due to the smaller number of rotamers. The less abundant species present for Na^+ , Na^+ -substrate and Na^+ -inhibitor bound Glt_{Tk} S281C had similar values to the most abundant species in apo Glt_{Tk} S281C, indicating the same spin label environment was present, which could correspond to the same conformation. Based on the number of rotamers in MMM predictions, the fastest $\tau_c \sim 6$ ns may correspond to an inward open state as this had the largest number of rotamers. In the cryo-EM structures an inward facing state was only observed for Na^+ and Na^+ -inhibitor bound Glt_{Tk}. The least abundant species in apo and most abundant species in Na^+ -substrate bound Glt_{Tk} had the slowest τ_c overall (~ 40 ns) which was different to the most abundant species in Na^+ and Na^+ -inhibitor bound (~ 20 ns). This may correspond to different outward facing conformations i.e. occluded vs open. Based on MMM, outward occluded Glt_{Tk} S281C had the smaller number of rotamers and is therefore likely to correspond to the slower τ_c . This was also the conformation present in the Na^+ -substrate bound cryo-EM structure.

3.4.6.2 Simulation cw-EPR spectra of Glt_{Tk} S281C in more biologically relevant environments

The same simulation methodology used for detergent micelles was applied to the closer-to-native environments studied for Glt_{Tk} S281C - the main difference being the absence of LT Q-band cw-EPR spectra. From LT X-band spectra the A_{zz} can be resolved from the distance between the two outermost peaks in the spectrum ($2A_{zz}$), in addition to the g_{zz} . The output results from LT Q-band spectral simulations of Glt_{Tk} S281C in detergent micelles were used as input values for the other membrane mimetics using the corresponding binding states. This assumed that the same cysteine variant had the same spin labelled position with respect to the rest of the protein, and therefore the previous g - and A -values were representative of the variant as a whole. The g - and A -values were kept fixed, varying only $lwpp$ which resulted in a good replication of the experimental LT X-band spectra. The results for the A - and g - values among the four environments were similar, further confirming the broadening and line shape changes observed at RT were a result of τ_c . The simulation

results for LT X-band cw-EPR spectra of Glt_{TK} S281C in proteoliposomes, SMALPs and nanodiscs are shown in Appendix A5. The spin Hamiltonian parameters of *g* and *A* extracted from LT X-band spectra were used as fixed parameters in simulations of RT X-band data. Optimising *lwpp*, τ_c and the relative proportions of the three species gave good quality representations of the RT spectra.

The results from RT simulations are in Table 3.6–Table 3.8 and Figure 3.15–Figure 3.17 for each of the membrane mimetics. High frequency (Q or W band) data may reveal minor perturbations of *g* and *A* values on changing the lipid environment, but X-band data is sufficient to demonstrate that the conformation of the apo protein or at least spin label mobility is identical in each of the membrane mimetics.

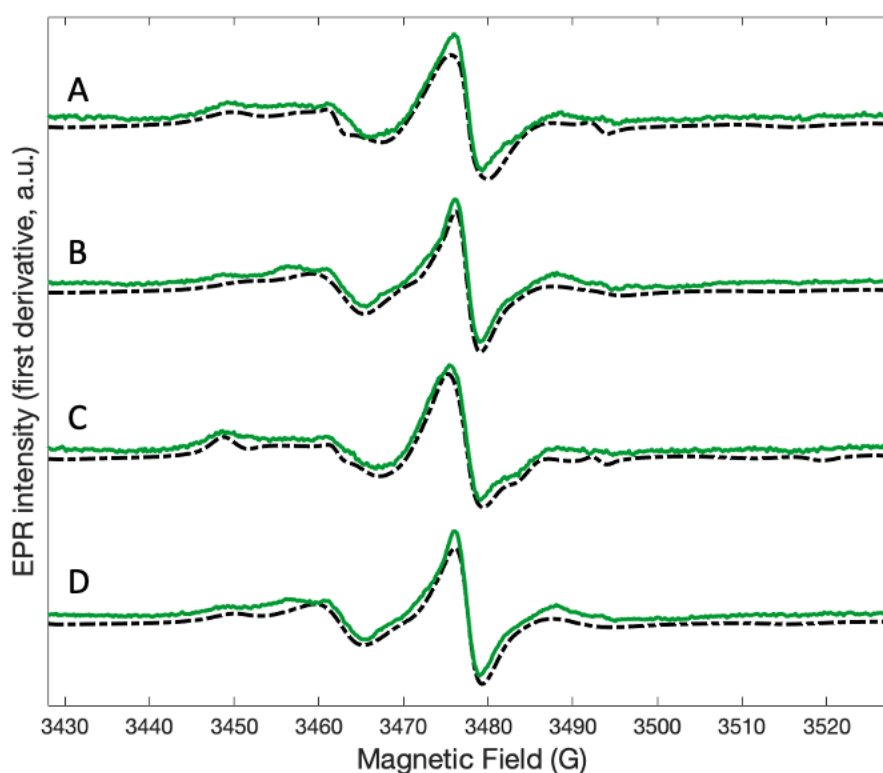


Figure 3.15 RT X-band cw-EPR spectra for Glt_{TK} S281C in proteoliposomes (green) and their respective simulations using EasySpin (black dashed line) for the proposed intermediates of the transport cycle: A) apo, B) Na⁺ (50 mM), C) substrate bound (50 mM Na⁺ and 1 mM L-aspartate) and D) inhibitor bound (50 mM Na⁺ and 1 mM DL-TBOA)

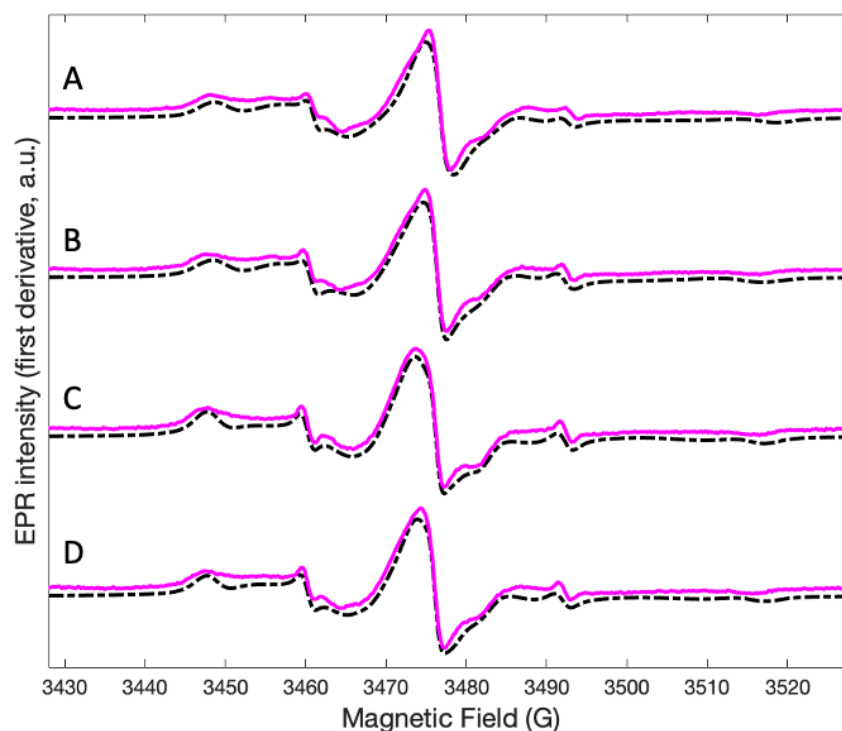


Figure 3.16 RT X-band cw-EPR spectra for Glt_{Tk} S281C in SMALPs (pink) and their respective simulations using EasySpin (black dashed line) for the proposed intermediates of the transport cycle: A) apo, B) Na⁺ (50 mM), C) substrate bound (50 mM Na⁺ and 1 mM L-aspartate) and D) inhibitor bound (50 mM Na⁺ and 1 mM DL-TBOA)

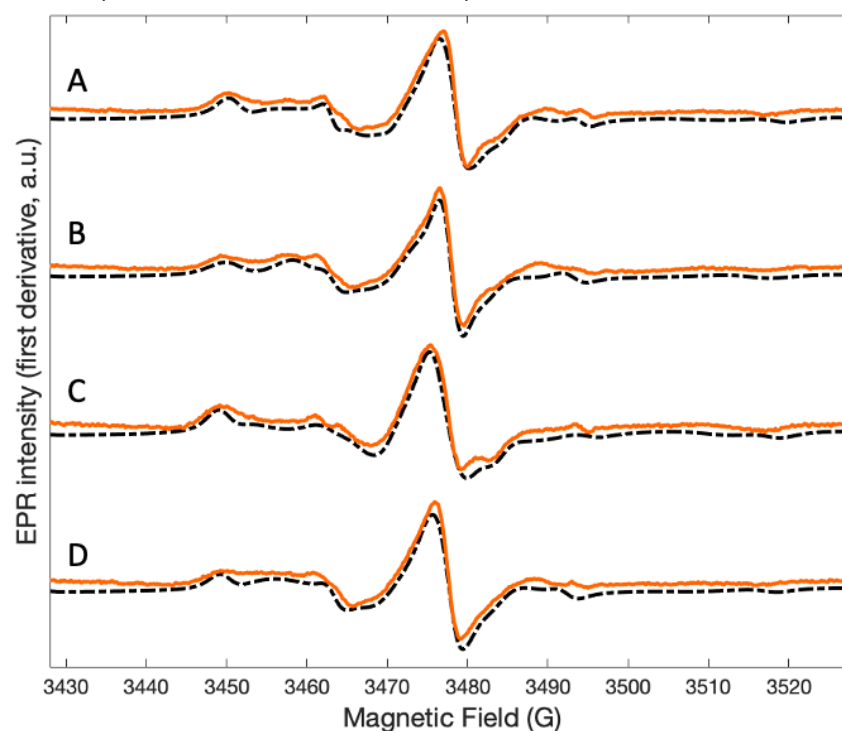


Figure 3.17 RT X-band cw-EPR spectra for Glt_{Tk} S281C in nanodiscs (orange) and their respective simulations using EasySpin (black dashed line) for the proposed intermediates of the transport cycle: A) apo, B) Na⁺ (50 mM), C) substrate bound (50 mM Na⁺ and 1 mM L-aspartate) and D) inhibitor bound (50 mM Na⁺ and 1 mM DL-TBOA)

	g_{zz}	g_{yy}	g_{xx}	A_{xx} (MHz)	A_{yy} (MHz)	A_{zz} (MHz)	Lwpp (mT)	τ_c (ns)	Ratio
Apo	2.0078	2.0054	2.0015	16.450	11.269	102.794	0.22	16.3	1
	2.0078	2.0054	2.0015	16.450	11.269	102.794	0.18	4.8	0.89
	2.0077	2.0050	2.0021	8.611	14.551	109.113	0.10	0.6	0.05
Na⁺	2.0078	2.0054	2.0015	17.457	11.472	102.720	0.18	4.5	1
	2.0078	2.0054	2.0015	17.457	11.472	102.720	0.22	21.1	0.82
	2.0077	2.0050	2.0021	8.611	14.551	109.113	0.10	0.6	0.05
Substrate	2.0078	2.0054	2.0016	15.784	10.336	102.792	0.20	20.5	1
	2.0078	2.0054	2.0016	15.784	10.336	102.792	0.19	3.7	0.33
	2.0077	2.0050	2.0021	8.611	14.551	109.113	0.10	0.6	0.01
Inhibitor	2.0078	2.0055	2.0016	15.364	12.139	102.797	0.16	3.7	1
	2.0078	2.0055	2.0016	15.364	12.140	102.797	0.18	17.5	0.58
	2.0077	2.0050	2.0021	8.611	14.551	109.113	0.10	0.6	0.01

Table 3.6 Best fit simulation values for RT X-band cw-EPR spectra of Glt_{TK} S281C in proteoliposomes. Results are shown for the four binding states with RMSD values of 0.0381 (apo), 0.0234 (Na⁺), 0.0259 (Na⁺ and L-aspartate), and 0.0280 (Na⁺ and DL-TBOA). The x, y, and z components of the g- and A-tensor and the linewidth (peak to peak, lwpp) are shown for each.

	g_{zz}	g_{yy}	g_{xx}	A_{xx} (MHz)	A_{yy} (MHz)	A_{zz} (MHz)	Lwpp (mT)	τ_c (ns)	Ratio
Apo	2.0078	2.0054	2.0015	16.450	11.269	102.794	0.18	5.5	1
	2.0078	2.0054	2.0015	16.450	11.269	102.794	0.23	55.7	0.60
	2.0077	2.0050	2.0021	8.611	14.551	109.113	0.10	0.6	0.03
Na⁺	2.0078	2.0054	2.0015	17.457	11.472	102.720	0.19	7.1	1
	2.0078	2.0054	2.0015	17.457	11.472	102.720	0.21	50.5	0.90
	2.0077	2.0050	2.0021	8.611	14.551	109.113	0.10	0.6	0.07
Substrate	2.0078	2.0054	2.0016	15.784	10.336	102.792	0.20	3.6	1
	2.0078	2.0054	2.0016	15.784	10.336	102.792	0.22	46.5	0.47
	2.0077	2.0050	2.0021	8.611	14.551	109.113	0.10	0.6	0.04
Inhibitor	2.0078	2.0055	2.0016	15.364	12.139	102.797	0.21	8.4	1
	2.0078	2.0055	2.0016	15.364	12.140	102.797	0.22	23.5	0.43
	2.0077	2.0050	2.0021	8.611	14.551	109.113	0.10	0.6	0.04

Table 3.7 Best fit simulation values for RT X-band cw-EPR spectra of GlT_{TK} S281C in SMALPs. Results are shown for the four binding states with RMSD values of 0.0248 (apo), 0.0204 (Na⁺), 0.0208 (Na⁺ and L-aspartate), and 0.0228 (Na⁺ and DL-TBOA). The x, y, and x components of the g- and A-tensor and the linewidth (peak to peak, lwpp) are shown for each.

	g_{zz}	g_{yy}	g_{xx}	A_{xx} (MHz)	A_{yy} (MHz)	A_{zz} (MHz)	Lwpp (mT)	τ_c (ns)	Ratio
Apo	2.0078	2.0054	2.0015	16.450	11.269	102.794	0.17	2.9	1
	2.0078	2.0054	2.0015	16.450	11.269	102.794	0.18	19.8	0.43
	2.0077	2.0050	2.0021	8.611	14.551	109.113	0.10	0.6	0.03
Na⁺	2.0078	2.0054	2.0015	17.457	11.472	102.720	0.18	10.0	1
	2.0078	2.0054	2.0015	17.457	11.472	102.720	0.23	42.2	0.91
	2.0077	2.0050	2.0021	8.611	14.551	109.113	0.10	0.6	0.07
Substrate	2.0078	2.0054	2.0016	15.784	10.336	102.792	0.22	42.7	1
	2.0078	2.0054	2.0016	15.784	10.336	102.792	0.18	2.3	0.41
	2.0077	2.0050	2.0021	8.611	14.551	109.113	0.10	0.6	0.02
Inhibitor	2.0078	2.0055	2.0016	15.364	12.139	102.797	0.20	5.8	1
	2.0078	2.0055	2.0016	15.364	12.140	102.797	0.21	45.5	0.36
	2.0077	2.0050	2.0021	8.611	14.551	109.113	0.10	0.6	0.04

Table 3.8 Best fit simulation values for RT X-band cw-EPR spectra of GlT_{TK} S281C in nanodiscs. Results are shown for the four binding states with RMSD values of 0.0252 (apo), 0.0258 (Na⁺), 0.0322 (Na⁺ and L-aspartate), and 0.0294 (Na⁺ and DL-TBOA). The x, y, and z components of the g- and A-tensor and the linewidth (peak to peak, lwpp) are shown for each.

The results in Table 3.6–Table 3.8 for spin-labelled Glt_{TK} S281C in detergent, proteoliposomes, SMALPs and nanodiscs highlights the effect of the environment on the protein. There is no particular environment which perfectly correlates with the other, showing the dynamic interactions of the protein at RT in the different binding states. Based on MMM predictions faster τ_c s (<10 ns) correspond with an inward facing conformation, with the slower τ_c s representing an outward facing conformations, and the larger values corresponding to an occluded state due to the significantly lower number of rotamers predicted by MMM. The highest τ_c obtained for proteoliposomes was 21.1 ns, none of the ~40 ns species in the other three environments was observed for any binding state. This could correlate to the outward occluded conformation not being present in the samples.

In an apo state, the most similar protein ratios observed are for detergent micelles and nanodiscs, with the most abundant species having the faster τ_c . However, SMALP exhibits the most similar τ_c to detergent (in the apo-state) but in different ratios. For detergent a ratio of 1:0.32 (with τ_c of 6.5:37.9 ns) was determined, compared to 1:0.60 (with τ_c of 5.5:55.7 ns) in SMALPs. For Na⁺ bound Glt_{TK}, detergent micelle results correspond to an outward facing state as the most abundant protein species, with a less abundant inward open state. This was the reverse in the other membrane mimetics which exhibited τ_c s corresponding to an inward facing conformation as the most abundant species with a second species which could correspond to an outward occluded conformation.

In detergent micelles, Na⁺-substrate bound Glt_{TK} S281C simulations showed a ratio of 1:0.29 corresponding to outward occluded and inward open conformations respectively. In proteoliposomes and nanodiscs, a similar observation was made where the ratio changed from 0.29 to 0.33 and 0.41 respectively, with the same conformations proposed. However, in SMALPs the τ_c values indicated the most abundant species was an inward facing state with a minor outward facing population which is the reverse to the other membrane environments. The τ_c for proteoliposomes was also lower than that of the other environments and may

correspond to an outward open conformation as this value corresponds to those observed in detergent micelles.

For Na⁺-inhibitor bound Glt_{Tk} S281C different results were observed in detergent compared to the other three environments: the protein species with the smallest τ_c was most abundant but for detergent it was the largest τ_c . For proteoliposomes and SMALPs, the same conformations were present compared to detergent but in different ratios i.e. inward open and outward open conformations. Nanodiscs had the slowest τ_c observed for Na⁺-inhibitor bound Glt_{Tk} S281C for the less abundant protein species which corresponds to the outward occluded conformation, where the outward open conformation is proposed to be present in the other three environments. These results align with those of the crystal structure, with Na⁺-inhibitor bound and Na⁺ bound Glt_{Tk} exhibiting both inward and outward facing states. Overall, the ratio of free-MTSL present in the samples was relatively consistent, with the smallest proportion being observed in proteoliposomes. Further investigation into these conformational changes proposed to occur during the transport cycle was made using pulsed EPR distance determination (Section 4.2).

3.5 Studying the conformational changes of Glt_{Tk} D298C using cw-EPR spectroscopy

3.5.1 Functional studies of Glt_{Tk} D298C in detergent micelles and proteoliposomes using cw-EPR

D298 of Glt_{Tk} is located on the intracellular side of the protein so MTSL attached to the cysteine of D298C was used to investigate the wider movement of Glt_{Tk} during the transport cycle. D298C is positioned between HP1 and TM7 which are both involved in substrate and sodium ion binding. The cw-EPR spectroscopic studies previously discussed for Glt_{Tk} S281C were repeated for spin labelled Glt_{Tk} D298C in detergent micelles and proteoliposomes. The addition of Na⁺ and L-aspartate or DL-TBOA were used to follow the conformational changes and dynamics during the transport cycle. Both LT and RT X-band cw-EPR spectra were recorded for each

sample. LT X-band cw-EPR spectra can be seen in Appendix A6 with their respective simulations, and RT spectra in Figure 3.18.

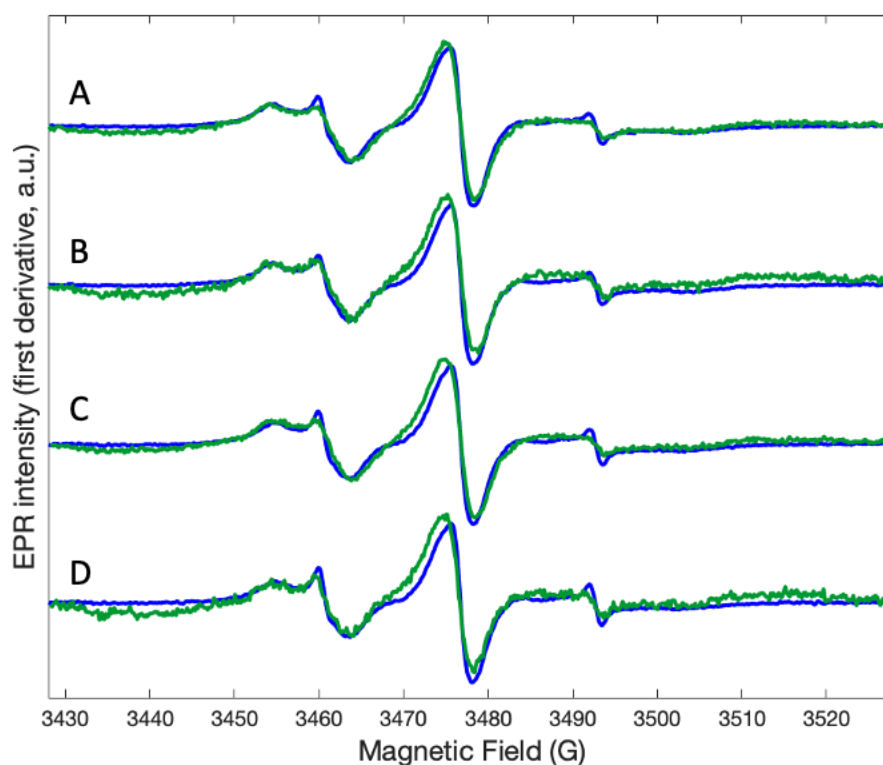


Figure 3.18 X-band RT cw-EPR spectra of Glt_{TK} D298C in detergent micelles (blue) and proteoliposomes (green) utilising substrate and co-transported Na⁺ to trap Glt_{TK} in proposed intermediates of the transport cycle: A) apo, B) Na⁺ (50 mM), C) substrate bound (50 mM Na⁺ and 1 mM L-aspartate) and D) inhibitor bound (50 mM Na⁺ and 1 mM DL-TBOA). Experimental parameters: microwave frequency = 9.758 GHz, centre field = 3478 G, sweep width = 120 G, modulation frequency = 100 kHz, modulation amplitude = 1–2 G, microwave power = 0.1997 mW, number averages = 64–100.

The overall general line shape of each binding condition in the two environments was very similar. It is clear proteoliposomes contain less residual free-MTSL compared to detergent micelles due to the extra wash steps in the reconstitution process - unless otherwise stated this was the case for all labelling positions. The main differences observed at RT were the noise levels and slight differences in the central peak width. The higher noise level is a result of the lower protein concentration obtained when reconstituted into proteoliposomes, which resulted in a baseline that was less flat. The differences in the width of the central peak could be a result of the different

modulation amplitudes; if too high this can cause artificial broadening which may be the case for the proteoliposome spectra. The modulation amplitude should not be wider than the distance between the peak and trough of the central peak. For all Glt_{TK} samples this was around 2 G, which was the modulation amplitude used to record cw-EPR spectra for the proteoliposome samples. Higher modulation amplitudes increase the signal intensity so for sample like proteoliposomes where it was hard to achieve a comparable signal intensity to detergent micelles (due to the lower protein concentration) it was beneficial to opt for a higher value. Spectra were also recorded at 1 G modulation amplitude to check the same line shapes as detergent micelles were observed, confirming the spectra were not over modulated.

The line shape of the detergent samples in the different binding states were very similar, with no obvious changes observed - this was the same for the proteoliposome samples too. This could indicate that either no or very subtle conformational changes occurred and therefore the spin-label did not experience a significant change in local environment; or the protein did not bind any substrate molecules added and so remained in an apo state. This was investigated further through simulations using EasySpin and pulsed EPR for distance determination.

3.5.2 Spectral simulations of Glt_{TK} D298C using EasySpin

EasySpin was used to extract numerical parameters from both LT and RT X-band cw-EPR spectra of Glt_{TK} D298C. The LT cw-EPR spectra were simulated using the g- and A-values from Glt_{TK} S281C Q-band simulations as start values. To obtain a close fit of the experimental data, the values were varied resulting in different results to Glt_{TK} S281C. Due to the absence of high frequency EPR data, the exact values extracted are not absolute and may differ. Therefore, values have been quoted to a lower degree of accuracy - this applies to all further variants too. For analysis of these simulation results the trend in the parameters will be focused on as opposed to the exact values. The results for the LT cw-EPR spectral simulations are shown in Appendix A6. The LT spectral simulation results were very similar, which was the first indication that the very subtle differences in line shape for the RT spectra were a result of τ_c . The LT

simulation results were then applied to the RT cw-EPR spectral simulations where the $lwpp$, τ_c and ratio of the species were varied within the defined limits. These again required 2 protein species and a residual free-MTSL species like Glt_{TK} S281C - the results are presented in Figure 3.19 and Table 3.9 for Glt_{TK} D298C in detergent micelles.

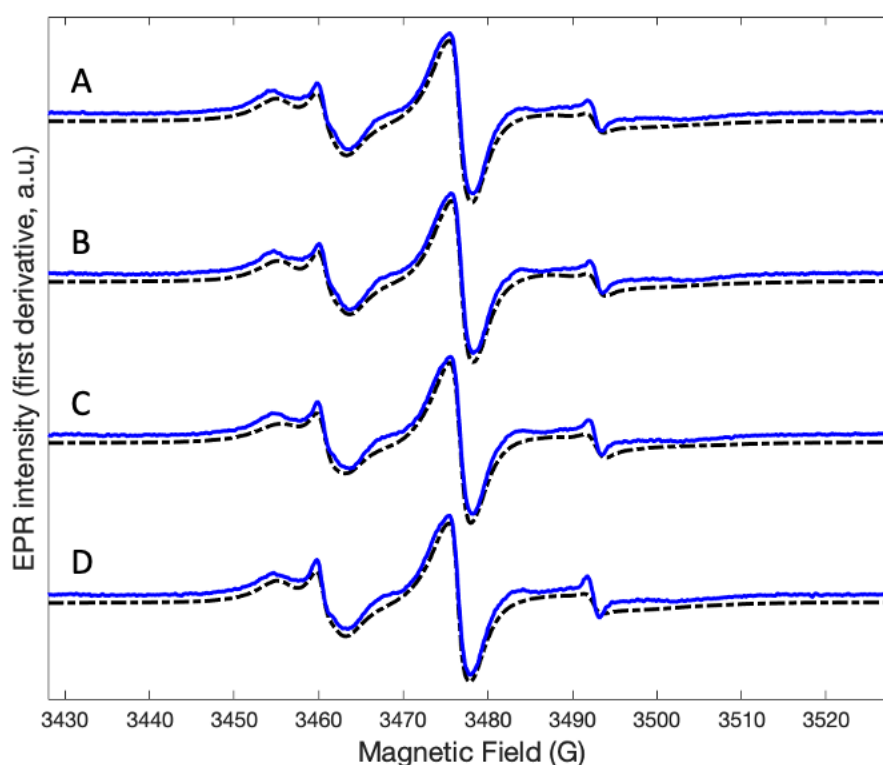


Figure 3.19 RT X-band cw-EPR spectra for Glt_{TK} D298C in detergent micelles (blue) and their respective simulations using EasySpin (black dashed line) for the proposed intermediates of the transport cycle: A) apo, B) Na⁺ (50 mM), C) substrate bound (50 mM Na⁺ and 1 mM L-aspartate) and D) inhibitor bound (50 mM Na⁺ and 1 mM DL-TBOA).

These results revealed less variance in τ_c compared to those observed for Glt_{TK} S281C, but values still range between 3.9 ns and 23.8 ns for the protein species. The slowest τ_c of 23.8 ns is seen for the apo state protein where it is believed to fluctuate between different conformational states. All other values were <12.2 ns which indicated fast molecular tumbling. This was unsurprising considering this is the most exposed variant to the intracellular environment meaning there is less steric hinderance to interfere with spin label mobility. For all binding states, the species with the faster τ_c was most abundant. For all except Na⁺-substrate bound, the τ_c was ~3 ns but

appeared slightly slower (6.3 ns) in the presence of L-aspartate. These could correspond to inward facing states based on this having the largest rotamer predictions in MMM. The second, less abundant protein species in the Na⁺-substrate bound state had similar τ_c value of 11.9 ns in a ratio of 1:0.67. This was the largest difference in ratio between the two protein species of all four binding states; apo and Na⁺ bound were almost a 1:1 ratio.

The slower τ_c of the Na⁺-substrate bound state has a similar value to the least abundant species in Na⁺ and Na⁺-inhibitor bound Glt_{TK} D298C (~11 ns) and may correspond to an intermediate outward occluded conformation, with the slower τ_c ~23.8 ns being an outward facing state. The similar τ_c s observed for the Na⁺-substrate bound state could represent similar conformational states such as intermediate outward occluded for 11.9 ns and intermediate inward occluded for the τ_c of 6.3 ns. The latter isn't present in the cryo-EM structures but inward facing Glt_{TK} D298C has fewer rotamers and a faster τ_c than outward facing. The ratio changes represent the protein moving between different conformational states during the transport cycle. So, although cw-EPR spectral line shapes looked similar, these results indicate the protein was undergoing some movement. This will be investigated further using pulsed EPR spectroscopy for Glt_{TK} D298C in detergent micelles.

	g_{zz}	g_{yy}	g_{xx}	A_{xx} (MHz)	A_{yy} (MHz)	A_{zz} (MHz)	Lwpp (mT)	τ_c (ns)	Ratio
Apo	2.0078	2.0057	2.0018	11.2	17.1	101.1	0.12	2.9	1
	2.0078	2.0057	2.0018	11.2	17.1	101.1	0.14	23.8	0.93
	2.0080	2.0046	2.0024	14.6	13.0	108.0	0.10	0.5	0.08
Na⁺	2.0078	2.0058	2.0017	15.2	17.4	101.1	0.16	2.8	1
	2.0078	2.0058	2.0017	15.2	17.4	101.1	0.14	12.2	0.99
	2.0080	2.0046	2.0024	14.6	13.0	108.0	0.09	0.5	0.10
Substrate	2.0078	2.0057	2.0017	15.6	16.1	101.1	0.12	6.3	1
	2.0078	2.0057	2.0017	15.6	16.1	101.1	0.14	11.9	0.67
	2.0080	2.0046	2.0024	14.6	13.0	108.0	0.10	0.5	0.15
Inhibitor	2.0078	2.0057	2.0018	16.6	15.9	101.1	0.14	3.5	1
	2.0078	2.0057	2.0018	16.6	15.9	101.1	0.10	10.7	0.84
	2.0080	2.0046	2.0024	14.6	13.0	108.0	0.08	0.5	0.07

Table 3.9 Best fit simulation values for RT X-band cw-EPR spectra of GlT_{TK} D298C in detergent micelles. Results are shown for the four binding states with RMSD values of 0.0196 (apo), 0.0197 (Na⁺), 0.0232 (Na⁺ and L-aspartate), and 0.0252 (Na⁺ and DL-TBOA). The x, y, and x components of the g- and A-tensor and the linewidth (peak to peak, lwpp) are shown for each.

LT X-band cw-EPR spectra of Glt_{7k} D298C reconstituted into proteoliposomes was simulated using the values for detergent micelles (Appendix A6) varying I_{wpp} . The fitting values from LT simulations were then applied to simulate the RT spectra where the results are shown in Table 3.10 and Figure 3.20. This applies to all other variants where spectra were simulated for protein in detergent micelles first followed by proteoliposomes.

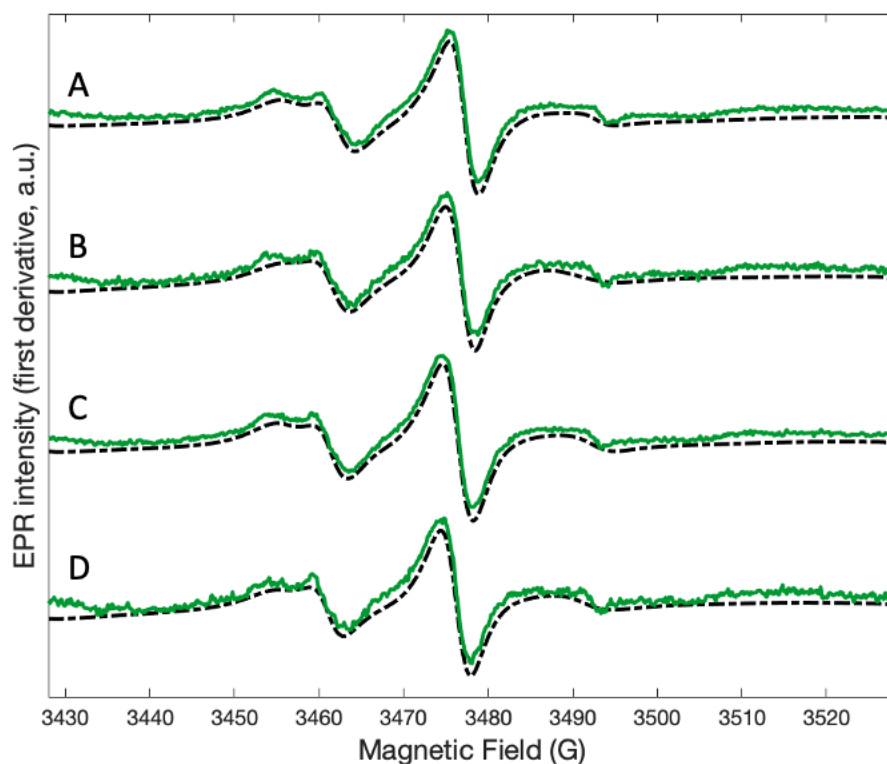


Figure 3.20 RT X-band cw-EPR spectra for Glt_{7k} D298C in proteoliposomes (green) and their respective simulations using EasySpin (black dashed line) for the proposed intermediates of the transport cycle: A) apo, B) Na⁺ (50 mM), C) substrate bound (50 mM Na⁺ and 1 mM L-aspartate) and D) inhibitor bound (50 mM Na⁺ and 1 mM DL-TBOA).

	g_{zz}	g_{yy}	g_{xx}	A_{xx} (MHz)	A_{yy} (MHz)	A_{zz} (MHz)	Lwpp (mT)	τ_c (ns)	Ratio
Apo	2.0078	2.0057	2.0018	11.2	17.1	101.1	0.13	6.9	1
	2.0078	2.0057	2.0018	11.2	17.1	101.1	0.11	12.3	0.93
	2.0080	2.0046	2.0024	14.6	13.0	108.0	0.10	0.5	0.06
Na⁺	2.0078	2.0058	2.0017	15.2	17.4	101.1	0.11	5.7	1
	2.0078	2.0058	2.0017	15.2	17.4	101.1	0.11	17.7	0.73
	2.0080	2.0046	2.0024	14.6	13.0	108.0	0.09	0.5	0.01
Substrate	2.0078	2.0057	2.0017	15.6	16.1	101.1	0.11	5.9	1
	2.0078	2.0057	2.0017	15.6	16.1	101.1	0.14	23.4	0.68
	2.0080	2.0046	2.0024	14.6	13.0	108.0	0.10	0.5	0.06
Inhibitor	2.0078	2.0057	2.0018	16.6	15.9	101.1	0.12	3.9	1
	2.0078	2.0057	2.0018	16.6	15.9	101.1	0.15	13.2	0.69
	2.0080	2.0046	2.0024	14.6	13.0	108.0	0.10	0.5	0.01

Table 3.10 Best fit simulation values for RT X-band cw-EPR spectra of Glt_{TK} D298C in proteoliposomes. Results are shown for the four binding states with RMSD values of 0.0297 (apo), 0.0459 (Na⁺), 0.0295 (Na⁺ and L-aspartate), and 0.0550 (Na⁺ and DL-TBOA). The x, y, and z components of the g- and A-tensor and the linewidth (peak to peak, lwpp) are shown for each

The simulation results between Glt_{TK} D298C in detergent micelles and proteoliposomes are similar, indicating the presence of the more native environment has not made much difference in the results observed for this residue. Like in detergent, all states had the most abundant protein species with the faster τ_c . This meant the spin label could rotate more freely and may be more solvent exposed. MMM results showed in inward facing states, MTSL has a greater rotational freedom hence a faster τ_c . In detergent micelles a $\tau_c \sim 3$ ns was proposed to correspond to an inward open conformation, which is present in the Na⁺-inhibitor proteoliposome sample. However, the τ_c in the other three binding states was ~ 6 ns as seen for Na⁺-substrate bound Glt_{TK} D298C in detergent micelles which was proposed to correspond to an intermediate conformation. The minor protein species in apo and Na⁺-inhibitor bound states corresponds to an intermediate outward occluded conformation, with the minor species for Na⁺ aligning with outward open (based on MMM predictions). This outward facing conformation is proposed to be the minor species for Na⁺-substrate Glt_{TK} D298C in proteoliposomes which had the slowest τ_c ; these results aligns with the cryo-EM structure conformation.

Glt_{TK} D298C had a smaller variance in τ_c compared to Glt_{TK} S281C. In proteoliposomes there is a larger difference in the τ_c of the Na⁺-substrate bound state (5.9 ns and 23.4 ns) indicating the two conformations sampled here are different to those in detergent micelles, with the most abundant species for both being inward facing, and the less abundant species as proposed to be an intermediate occluded state in detergent but outward open in proteoliposomes. Overall, there is a smaller proportion of free MTSL in the proteoliposome samples as a result of the additional wash steps, but the ratios between the two protein species in both environments are very similar, particularly for apo and Na⁺-substrate bound states.

3.6 Studying the conformational changes of Glt_{Tk} I312C using cw-EPR spectroscopy

3.6.1 Functional studies of Glt_{Tk} I312C in detergent micelles and proteoliposomes using cw-EPR

Glt_{Tk} I312C is situated just before the highly conserved NMDGT motif which is involved in binding of sodium ions and substrate molecules. Functional studies (at RT and LT) were conducted using cw-EPR spectroscopy of Glt_{Tk} I312C in both detergent micelles and proteoliposomes to investigate the intermediate states during the transport cycle. The LT spectra can be seen with their respective fits in Appendix A7. The similar line shape of the RT cw-EPR spectra between proteoliposomes and detergent micelles gave an initial indication that there the local environment of the spin label did not significantly vary between the two membrane mimetics (Figure 3.21). There were subtle changes in spectral line shape between proteoliposomes and detergent micelles for the different binding states, mostly for the position of the low field peak highlighted in Figure 3.21. These changes were characterised quantitatively using EasySpin and investigated further using pulsed EPR distance determination experiments.

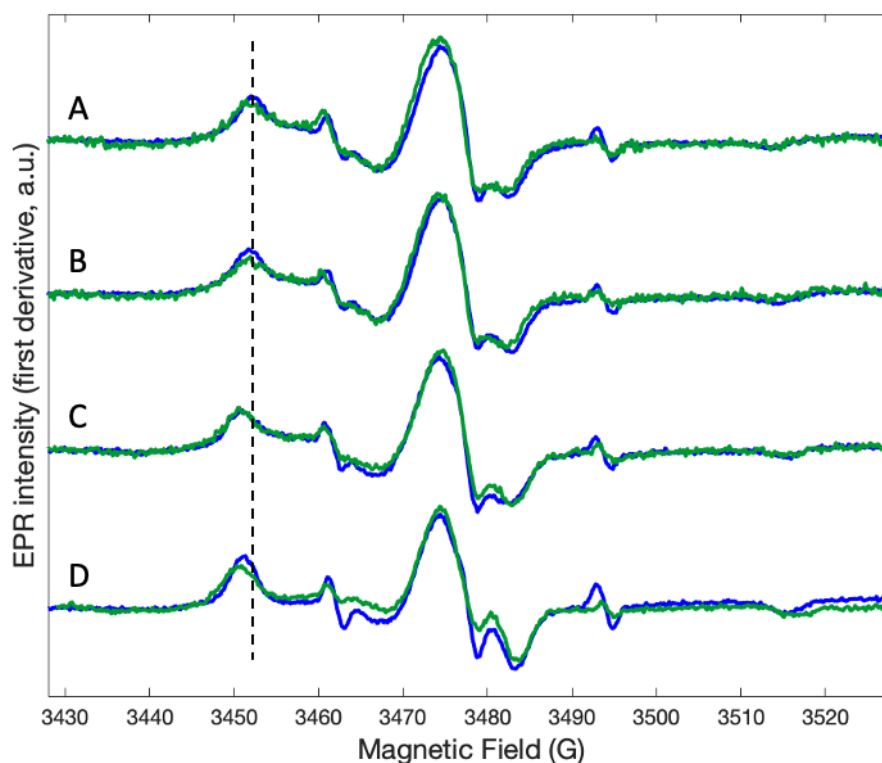


Figure 3.21 X-band RT cw-EPR spectra of Glt_{TK} I312C in detergent micelles (blue) and proteoliposomes (green) utilising substrate and co-transported Na⁺ to trap Glt_{TK} in proposed intermediates of the transport cycle: A) apo, B) Na⁺ (50 mM), C) substrate bound (50 mM Na⁺ and 1 mM L-aspartate) and D) inhibitor bound (50 mM Na⁺ and 1 mM DL-TBOA). Black dashed lines indicate regions of the spectra where subtle changes are observed. Experimental parameters: microwave frequency = 9.758 GHz, centre field = 3478 G, sweep width = 120 G, modulation frequency = 100 kHz, modulation amplitude = 1–2 G, microwave power = 0.1997 mW, number averages = 64–100.

3.6.2 Spectral simulations of Glt_{TK} I312C using EasySpin

LT cw-EPR spectra of detergent purified Glt_{TK} I312C showed very little variation in the EasySpin simulation values which is also reflected by the similarity in spectral line shape (Appendix A7). This means the subtle changes in spectral line shape observed in RT spectra were a result of τ_c . The results are presented in Figure 3.22 and Table 3.11.

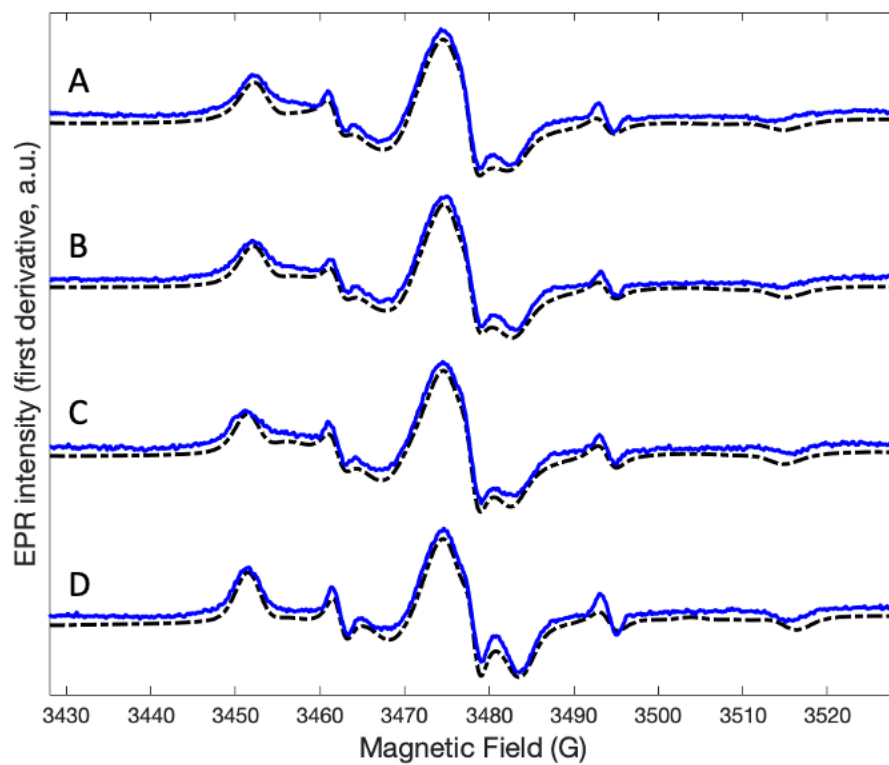


Figure 3.22 RT X-band cw-EPR spectra for Glt_{TK} I312C in detergent micelles (blue) and their respective simulations using EasySpin (black dashed line) for the proposed intermediates of the transport cycle: A) apo, B) Na⁺ (50 mM), C) substrate bound (50 mM Na⁺ and 1 mM L-aspartate) and D) inhibitor bound (50 mM Na⁺ and 1 mM DL-TBOA).

	g_{zz}	g_{yy}	g_{xx}	A_{xx} (MHz)	A_{yy} (MHz)	A_{zz} (MHz)	Lwpp (mT)	τ_c (ns)	Ratio
Apo	2.0081	2.0054	2.0015	15.3	12.1	92.9	0.26	8.7	1
	2.0081	2.0054	2.0015	15.3	12.1	92.9	0.20	51.7	0.80
	2.0080	2.0046	2.0020	9.7	13.0	106.2	0.11	0.5	0.05
Na⁺	2.0081	2.0055	2.0015	15.7	13.0	92.9	0.28	11.5	1
	2.0081	2.0055	2.0015	15.7	13.0	92.9	0.22	24.1	0.76
	2.0080	2.0046	2.0020	9.7	13.0	106.2	0.13	0.5	0.06
Substrate	2.0081	2.0054	2.0014	14.3	12.3	92.9	0.22	12.6	1
	2.0081	2.0054	2.0014	14.3	12.3	92.9	0.23	38.9	0.76
	2.0080	2.0046	2.0020	9.7	13.0	106.2	0.13	0.5	0.07
Inhibitor	2.0081	2.0056	2.0015	15.2	14.4	92.9	0.26	53.8	1
	2.0081	2.0056	2.0015	15.2	14.4	92.9	0.21	8.3	0.79
	2.0080	2.0046	2.0020	9.7	13.0	106.2	0.11	0.5	0.08

Table 3.11 Best fit simulation values for RT X-band cw-EPR spectra of GlT_{rk} I312C in detergent micelles. Results are shown for the four binding states with RMSD values of 0.0266 (apo), 0.0286 (Na⁺), 0.0274 (Na⁺ and L-aspartate), and 0.0329 (Na⁺ and DL-TBOA). The x, y, and x components of the g- and A-tensor and the linewidth (peak to peak, lwpp) are shown for each.

The EasySpin simulation results of Glt_{7k} I312C at LT are presented in Appendix A7. For all four binding states of Glt_{7k} I312C, the EasySpin fit was achieved using two spin labelled protein species in addition to a free MTSL species. The ratio between the two protein species was similar for all binding states at ~ 1:0.8. For the apo, Na⁺ and Na⁺-substrate bound spectra, the most abundant species had the faster τ_c ~10 ns. *In silico* predictions based on MMM showed outward facing conformations had a higher number of rotamers than inward facing, which would indicate a shorter τ_c . Therefore, this species is proposed to correspond to an outward facing conformation. For Na⁺-inhibitor bound Glt_{7k} I312C the most abundant species had a slower τ_c 's of 53.8 ns which corresponds to the minor species in the apo state. When comparing these results to the predicted rotamers in MMM, this τ_c may correspond to an inward facing state, with intermediary τ_c s of 24.1 ns (Na⁺ bound) and 38.9 ns (Na⁺-substrate bound) proposed to be occluded states. The results for Na⁺-inhibitor bound Glt_{7k} I312C are almost exactly the reverse for those of apo in terms of both τ_c and the ratio which may mean the same two conformational states were present but in different ratios.

	g_{zz}	g_{yy}	g_{xx}	A_{xx} (MHz)	A_{yy} (MHz)	A_{zz} (MHz)	Lwpp (mT)	τ_c (ns)	Ratio
Apo	2.0081	2.0054	2.0015	15.3	12.1	92.9	0.21	12.3	1
	2.0081	2.0054	2.0015	15.3	12.1	92.9	0.26	29.2	0.57
	2.0080	2.0046	2.0020	9.7	13.0	106.2	0.14	0.5	0.04
Na⁺	2.0081	2.0055	2.0015	15.7	13.0	92.9	0.15	13.2	1
	2.0081	2.0055	2.0015	15.7	13.0	92.9	0.27	26.3	0.76
	2.0080	2.0046	2.0020	9.7	13.0	106.2	0.13	0.5	0.03
Substrate	2.0081	2.0054	2.0014	14.3	12.3	92.9	0.22	11.1	1
	2.0081	2.0054	2.0014	14.3	12.3	92.9	0.29	71.4	0.99
	2.0080	2.0046	2.0020	9.7	13.0	106.2	0.16	0.5	0.05
Inhibitor	2.0081	2.0056	2.0015	15.2	14.4	92.9	0.29	55.2	1
	2.0081	2.0056	2.0015	15.2	14.4	92.9	0.21	22.1	0.88
	2.0080	2.0046	2.0020	9.7	13.0	106.2	0.12	0.5	0.03

Table 3.12 Best fit simulation values for RT X-band cw-EPR spectra of GlT_{TK}I312C in proteoliposomes. Results are shown for the four binding states with RMSD values of 0.0388 (apo), 0.0396 (Na⁺), 0.0343 (Na⁺ and L-aspartate), and 0.0452 (Na⁺ and DL-TBOA). The x, y, and x components of the g- and A-tensor and the linewidth (peak to peak, lwpp) are shown for each.

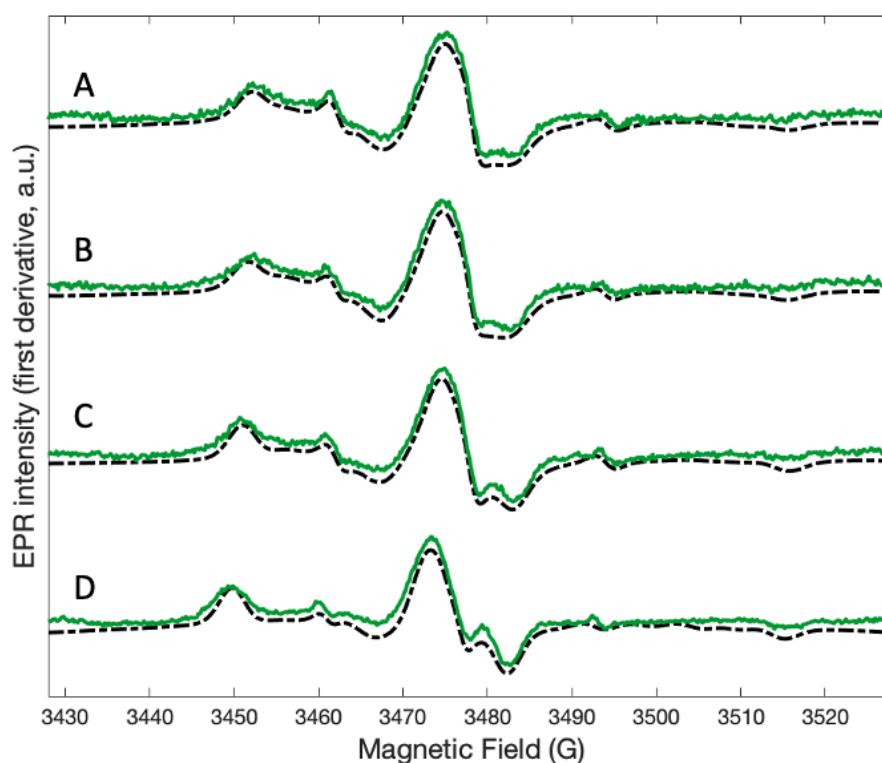


Figure 3.23 RT X-band cw-EPR spectra for Glt_{TK} I312C in proteoliposomes (green) and their respective simulations using EasySpin (black dashed line) for the proposed intermediates of the transport cycle: A) apo, B) Na⁺ (50 mM), C) substrate bound (50 mM Na⁺ and 1 mM L-aspartate) and D) inhibitor bound (50 mM Na⁺ and 1 mM DL-TBOA).

The EasySpin simulation results for Glt_{TK} I312C in proteoliposomes (Figure 3.23 and Table 3.12) varied slightly compared to detergent micelles, despite the spectra appearing to be very similar. This may be a result of the high noise in the liposome spectra due to lower protein concentration which EasySpin could have interpreted as line shape rather than noise. The biggest contrast in results was for apo and Na⁺-substrate bound samples where apo Glt_{TK} I312C in detergent micelles had a ratio of 1:0.80 with τ_c s of 8.7 ns and 51.7 ns, and in proteoliposomes this changed to a 1:0.57 ratio with τ_c s of 12.3 ns and 29.2 ns. Despite different τ_c s between the two environments, the faster τ_c was the most abundant species corresponding to an outward facing conformation. In an apo state the protein can sample a range of conformations which is exhibited by these results. The minor species for apo Glt_{TK} I312C proteoliposomes is similar to τ_c s assigned as intermediate occluded conformations in detergent micelles. Whereas in detergent the minor species was

proposed to be an inward facing conformation. This difference in τ_c could be the difference between intermediate inward occluded and inward open.

EasySpin simulation results for Na⁺ bound Glt_{TK} I312C in proteoliposomes were similar to detergent micelles where the outward facing state was assigned as the major species, and an intermediate outward occluded as the minor species. When Na⁺-substrate bound, the most abundant species had a $\tau_c \sim 12$ ns proposed to be an outward facing conformation which was also observed in detergent micelles. However, the less abundant species had a slower τ_c in proteoliposomes (38.9 ns for detergent, and 71.4 ns for proteoliposomes). Based on MMM predictions, this could correspond to the inward facing conformation which had $\tau_c \sim 50$ ns in detergent micelles. This may indicate the presence of the lipid bilayer in the proteoliposomes has either revealed a different conformational state not observed in detergent micelles or caused a much tighter environment of the spin label in the same protein conformation. The results Na⁺-inhibitor bound Glt_{TK} I312C were similar between the two environments - with the only difference being the τ_c of the faster, less abundant species (at 22.1 ns) which may represent an outward occluded conformation as opposed to the outward facing state seen in detergent micelles.

3.7 Studying the conformational changes of Glt_{TK} M314C using cw-EPR spectroscopy

3.7.1 Functional studies of Glt_{TK} M314C in detergent micelles and proteoliposomes using cw-EPR

Glt_{TK} M314C is part of the highly conserved NMDGT motif which is directly involved in binding sodium ions and substrate. EPR spectra were recorded at RT and LT for Glt_{TK} M314C in detergent micelles and proteoliposomes (shown in Appendix A8 and A9). The LT cw-EPR spectra had a similar line shapes in the four binding states used to follow the conformational changes during the transport cycle. The LT proteoliposome spectra had the shoulder on the central peak, as previously discussed for S281C, which is believed to be trapped oxygen.

The RT spectra for Glt_{TK} M314C in detergent micelles revealed line shape changes for the different binding states (Figure 3.24). These were more pronounced than Glt_{TK} D298C and I312C presented previously, indicating a bigger change in the relative environment of the spin label which could correspond to larger scale conformational changes. The predictions from MMM indicated this residue undergoes movements of ~2.5 nm during the transport cycle. This contrasts significantly to Glt_{TK} I312C where very small changes in line shape for RT cw-EPR spectra were observed, and only 1 nm distance changes were predicted. There was more free-MTSL present in the RT cw-EPR spectra, which correlates with the low labelling efficiency (44%, Table 3.2) and also the lowest number of rotamers from MMM indicating it is the least accessible residue.

As with all Glt_{TK} variants, the proteoliposome spectra had a lower proportion of free-MTSL but otherwise exhibited the same line shapes as detergent micelles (Appendix A8). The lower protein concentration in proteoliposomes resulted in higher noise levels in the spectral line shape, which was also slightly broadened at the central peak compared to the detergent micelle spectra due to the modulation amplitude selected to achieve a better signal to noise ratio (as previously discussed for Glt_{TK} D298C). The line shape changes were quantified using EasySpin and investigated further using pulsed EPR distance determination experiments.

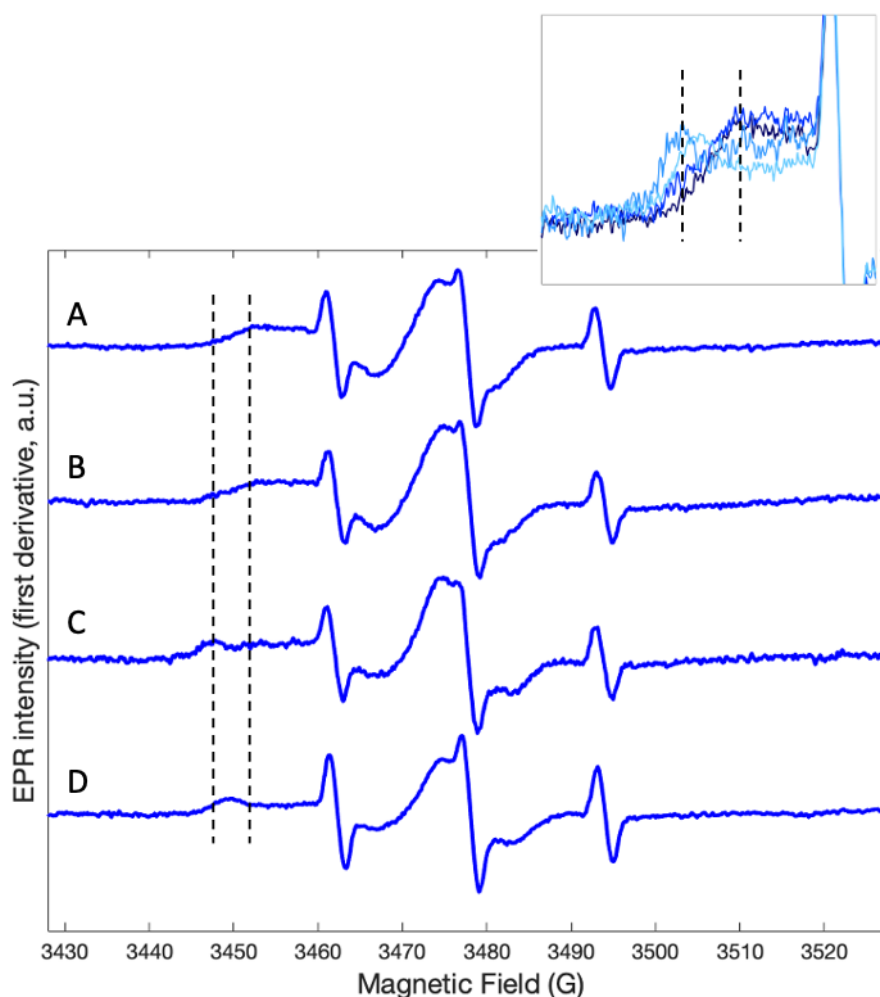


Figure 3.24 X-band RT cw-EPR spectra of Glt_{Tk} M314C in detergent micelles utilising substrate and co-transported Na⁺ to trap Glt_{Tk} in proposed intermediates of the transport cycle: A) apo, B) Na⁺ (50 mM), C) substrate bound (50 mM Na⁺ and 1 mM L-aspartate) and D) inhibitor bound (50 mM Na⁺ and 1 mM DL-TBOA). Black dashed lines indicate regions of the spectra where subtle changes are observed (see insert). Zoomed in area of the biggest shape changes in the binding conditions shown darkest to lightest for A) to D). Experimental parameters: microwave frequency = 9.758 GHz, centre field = 3478 G, sweep width = 120 G, modulation frequency = 100 kHz, modulation amplitude = 1G, microwave power = 0.1997 mW, number averages = 64.

3.7.2 Spectral simulations of Glt_{Tk} M314C using EasySpin

The simulation results of the LT spectra for Glt_{Tk} M314C in detergent micelles is shown in Appendix A9.1. Simulation of the RT spectra (presented in Figure 3.25 and Table 3.13) quantified the excess free-MTSL present in these samples (~7%) which is larger than all of the other Glt_{Tk} variants. For all binding states except Na⁺ bound, the most abundant protein species had a faster τ_c (~10 ns) and the less abundant species had

a $\tau_c \sim 30$ ns for apo and Na⁺-substrate, and 52.0 ns for Na⁺-inhibitor. This means the same conformations may be present in apo and Na⁺-substrate bound but in slightly different ratios, with the same most abundant species for Na⁺-inhibitor bound Glt_{TK} M314C but a different less abundant conformation. 52 ns was the slowest τ_c observed for Glt_{TK} M314C in detergent micelles which could be a result of the DL-TBOA in the substrate binding site restricting spin label motion because it is bulkier than L-aspartate. Na⁺-bound Glt_{TK} M314C had the similar τ_c s to the two species in apo and Na⁺-substrate simulations, but in opposite ratios - apo had τ_c s of 6.3 ns and 26.2 ns in a ratio of 1:0.67, contrasting to Na⁺-bound τ_c s of 34.6 ns and 4.5 ns in a ratio of 1:0.66. This is likely to represent the same conformations in different proportions. It is difficult to assign τ_c values to specific conformations due to the similar, low number of predicted rotamers from *in silico* analysis in the different conformational states. Overall, a mixture of conformations is suggested for each binding state.

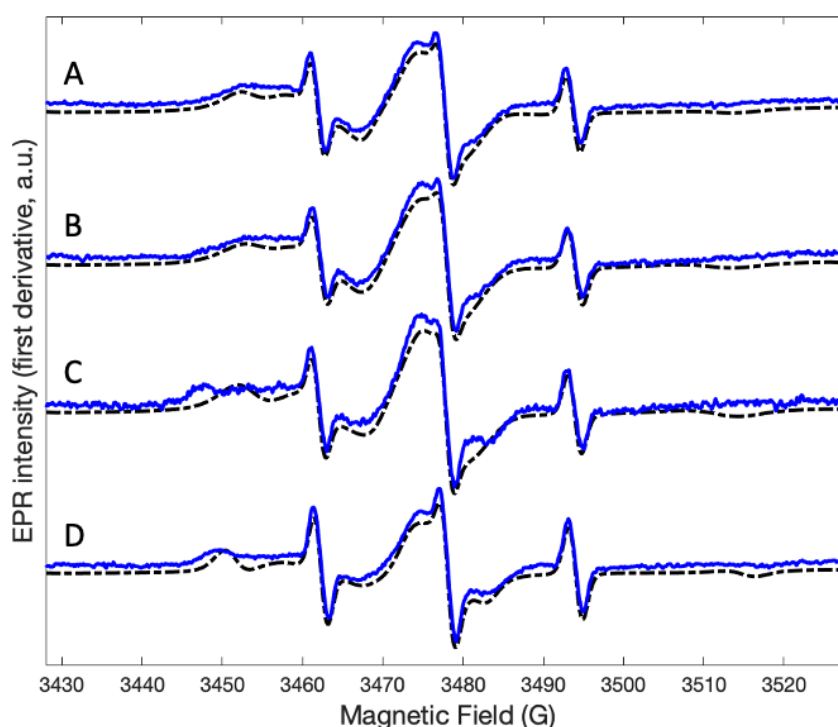


Figure 3.25 RT X-band cw-EPR spectra for Glt_{TK} M314C in detergent micelles (blue) and their respective simulations using EasySpin (black dashed line) for the proposed intermediates of the transport cycle: A) apo, B) Na⁺ (50 mM), C) substrate bound (50 mM Na⁺ and 1 mM L-aspartate) and D) inhibitor bound (50 mM Na⁺ and 1 mM DL-TBOA).

	g_{zz}	g_{yy}	g_{xx}	A_{xx} (MHz)	A_{yy} (MHz)	A_{zz} (MHz)	Lwpp (mT)	τ_c (ns)	Ratio
Apo	2.0078	2.0058	2.0016	13.6	15.5	96.1	0.16	6.3	1
	2.0078	2.0058	2.0016	13.6	15.5	96.1	0.21	26.2	0.67
	2.0080	2.0046	2.0020	9.7	13.0	106.2	0.17	0.4	0.11
Na⁺	2.0078	2.0059	2.0017	13.3	16.5	96.1	0.22	34.6	1
	2.0078	2.0059	2.0017	13.3	16.5	96.1	0.18	4.5	0.66
	2.0080	2.0046	2.0020	9.7	13.0	106.2	0.18	0.4	0.10
Substrate	2.0078	2.0058	2.0016	14.8	15.5	96.1	0.22	8.9	1
	2.0078	2.0058	2.0016	14.8	15.5	96.1	0.20	31.8	0.49
	2.0080	2.0046	2.0020	9.7	13.0	106.2	0.16	0.4	0.10
Inhibitor	2.0078	2.0059	2.0017	14.5	15.8	96.1	0.19	11.8	1
	2.0078	2.0059	2.0017	14.5	15.8	96.1	0.22	52.0	0.80
	2.0080	2.0046	2.0020	9.7	13.0	106.2	0.17	0.4	0.12

Table 3.13 Best fit simulation values for RT X-band cw-EPR spectra of Glt_{TK} M314C in detergent micelles. Results are shown for the four binding states with RMSD values of 0.0259 (apo), 0.0392 (Na⁺), 0.0211 (Na⁺ and L-aspartate), and 0.0318 (Na⁺ and DL-TBOA). The x, y, and x components of the g- and A-tensor and the linewidth (peak to peak, lwpp) are shown for each.

Simulations of LT spectra for Glt_{TK} M314C in proteoliposomes (Appendix A9.2) showed very similar values for each binding state. This confirmed the changes in line shape seen in the RT cw-EPR spectra were from τ_c values and a change in membrane mimetic did not have a big effect on polarity. The results of RT simulations of Glt_{TK} M314C in proteoliposomes are shown in Figure 3.26 and Table 3.14.

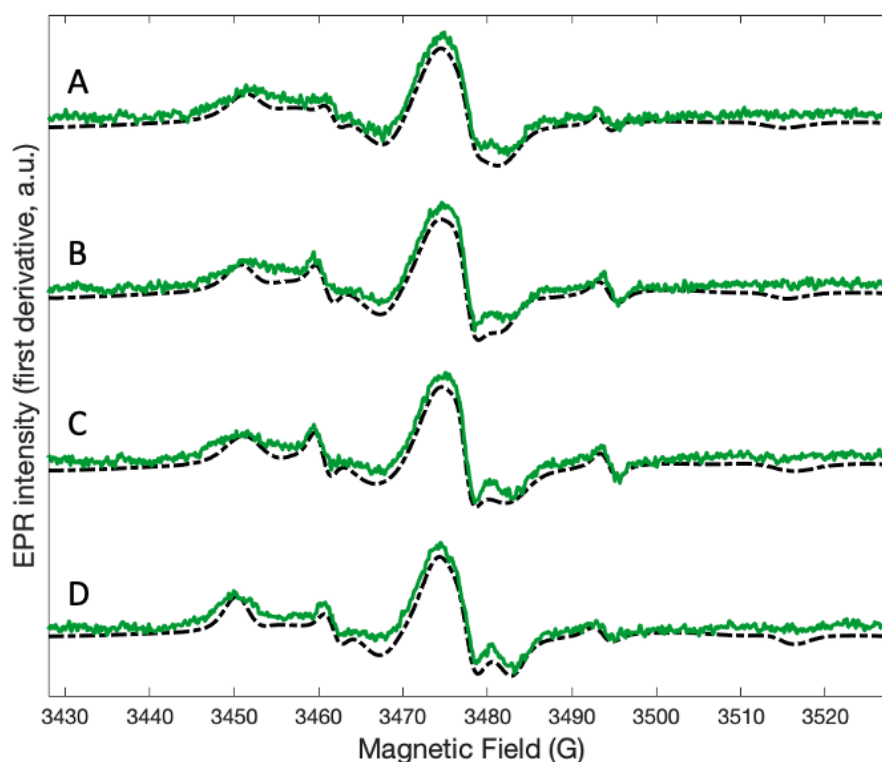


Figure 3.26 RT X-band cw-EPR spectra for Glt_{TK} M314C in proteoliposomes (green) and their respective simulations using EasySpin (black dashed line) for the proposed intermediates of the transport cycle: A) apo, B) Na⁺ (50 mM), C) substrate bound (50 mM Na⁺ and 1 mM L-aspartate) and D) inhibitor bound (50 mM Na⁺ and 1 mM DL-TBOA).

	g_{zz}	g_{yy}	g_{xx}	A_{xx} (MHz)	A_{yy} (MHz)	A_{zz} (MHz)	Lwpp (mT)	τ_c (ns)	Ratio
Apo	2.0078	2.0058	2.0016	13.6	15.5	96.1	0.20	11.6	1
	2.0078	2.0058	2.0016	13.6	15.5	96.1	0.22	38.1	0.80
	2.0080	2.0046	2.0020	9.7	13.0	106.2	0.17	0.4	0.01
Na⁺	2.0078	2.0059	2.0017	13.3	16.5	96.1	0.21	50.1	1
	2.0078	2.0059	2.0017	13.3	16.5	96.1	0.20	8.6	0.70
	2.0080	2.0046	2.0020	9.7	13.0	106.2	0.18	0.4	0.06
Substrate	2.0078	2.0058	2.0016	14.8	15.5	96.1	0.15	3.2	1
	2.0078	2.0058	2.0016	14.8	15.5	96.1	0.22	39.9	0.86
	2.0080	2.0046	2.0020	9.7	13.0	106.2	0.16	0.4	0.07
Inhibitor	2.0078	2.0059	2.0017	14.5	15.8	96.1	0.22	7.5	1
	2.0078	2.0059	2.0017	14.5	15.8	96.1	0.19	53.9	0.95
	2.0080	2.0046	2.0020	9.7	13.0	106.2	0.17	0.4	0.05

Table 3.14 Best fit simulation values for RT X-band cw-EPR spectra of GlT_{TK} M314C in proteoliposomes. Results are shown for the four binding states with RMSD values of 0.0486 (apo), 0.0544 (Na⁺), 0.0477 (Na⁺ and L-aspartate), and 0.0513 (Na⁺ and DL-TBOA). The x, y, and x components of the g- and A-tensor and the linewidth (peak to peak, lwpp) are shown for each.

Compared to detergent micelles, the τ_c values obtained from EasySpin for proteoliposomes are generally slower which was also seen for Glt_{TK} I312C. This data was harder to fit due to the low signal to noise ratio of the spectra, in particular for Na⁺ bound. The slower τ_c may suggest the presence of the lipid bilayer particularly affected the residues around the binding sites but could also result from increased noise. The more most abundant species had the fastest τ_c in all binding states, except Na⁺ bound which had a slower τ_c for the most abundant species as seen in detergent micelles. Na⁺-Inhibitor bound Glt_{TK} M314C still had the slowest τ_c of all the binding conditions, but a similar value was also observed for the Na⁺ bound state. The conformational changes of Glt_{TK} M314C was further investigated using pulsed EPR experiments to follow distance changes. This variant was particularly challenging given the poor labelling efficiency in detergent, and the additional signal losses experienced when reconstituted.

3.8 Studying the conformational changes of Glt_{TK} H334C using cw-EPR spectroscopy

3.8.1 Functional studies of Glt_{TK} H334C in detergent micelles and proteoliposomes using cw-EPR

Glt_{TK} H334 is the most exposed variant to the extracellular environment. Functional studies of Glt_{TK} H334C in detergent micelles and proteoliposomes were recorded using cw-EPR spectroscopy at both RT and LT. The RT cw-EPR spectra had only subtle differences in line shape for both environments in each binding state. The main difference in line shape was the intensity of the low field peak where apo was the highest intensity, with Na⁺ and Na⁺-inhibitor bound Glt_{TK} H334C spectra appearing very similar. The main difference between the two environments was the increased level of residual free-MTSL in detergent micelles (Figure 3.27). MMM results had distance distributions spanning 3.5 nm indicating this residue undergoes large scale conformational changes. It is possible, with this variant being the most exposed to the extracellular environment, that the environment of the spin label remained

relatively constant even when the protein underwent conformational changes hence a large change in line shape was not seen.

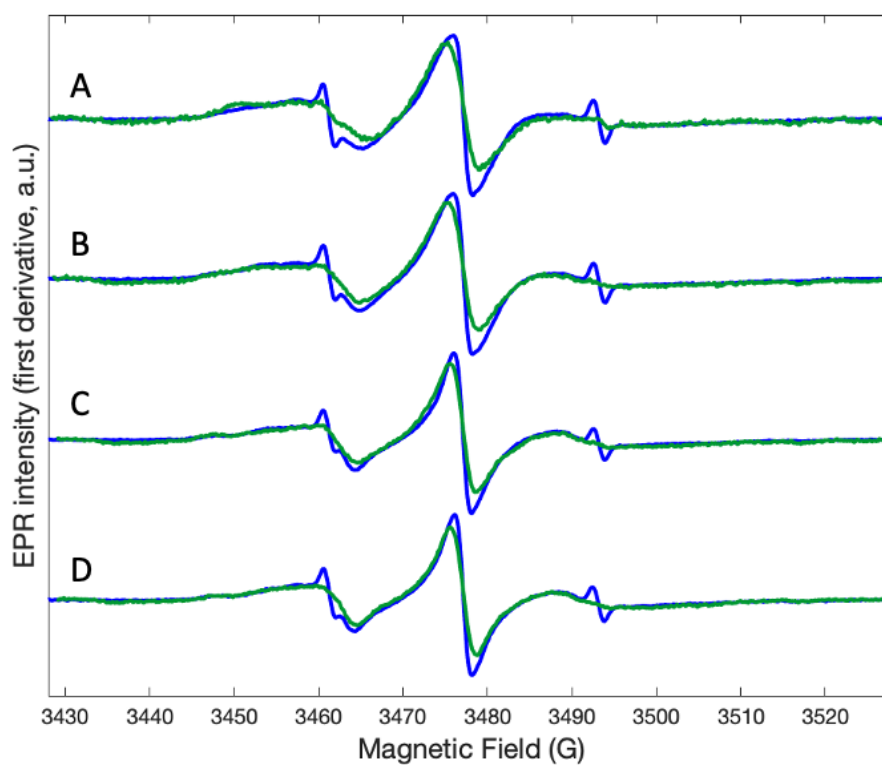


Figure 3.27 X-band RT cw-EPR spectra of Glt_{Tk} H334C in detergent micelles (blue) and proteoliposomes (green) utilising substrate and co-transported Na⁺ to trap Glt_{Tk} in proposed intermediates of the transport cycle: A) apo, B) Na⁺ (50 mM), C) substrate bound (50 mM Na⁺ and 1 mM L-aspartate) and D) inhibitor bound (50 mM Na⁺ and 1 mM DL-TBOA). Experimental parameters: microwave frequency = 9.758 GHz, centre field = 3478 G, sweep width = 120 G, modulation frequency = 100 kHz, modulation amplitude = 1–2 G, microwave power = 0.1997 mW, number averages = 64–100.

3.8.2 Spectral simulations of Glt_{Tk} H334C using EasySpin

To further investigate the very subtle changes in RT cw-EPR spectra, EasySpin was used to extract numerical parameters. The LT simulation results (presented in Appendix A10) showed similar results for all binding states, indicating the subtle changes seen in line shape of the RT spectra are a result of τ_c . The output values were used for simulation of RT spectra where the results can be seen in Figure 3.28 and Table 3.15 for Glt_{Tk} H334C in detergent micelles.

In all four binding states two protein species and a free-MTSL species were required, with the former differing in their linewidth, τ_c and relative ratios. The same protein species with a $\tau_c \sim 3.8$ ns was the most abundant species for each of the four binding states, likely to represent a single conformation of Glt_{TK} H334C. Na⁺ and Na⁺-inhibitor bound Glt_{TK} H334C also had similar τ_c s for the less abundant species which is reflected in their similar spectral line shapes. This was only fractionally slower than the most abundant species so could represent the difference between open and occluded conformations. Apo Glt_{TK} H334C had the slowest value ~ 30 ns, and Na⁺-substrate had a less abundant species with τ_c of 20.6 ns which are likely to correspond to additional conformational states. It is difficult to assign specific conformational states to these results as there is overlap between the MMM rotamer predictions of inward and outward facing conformations.

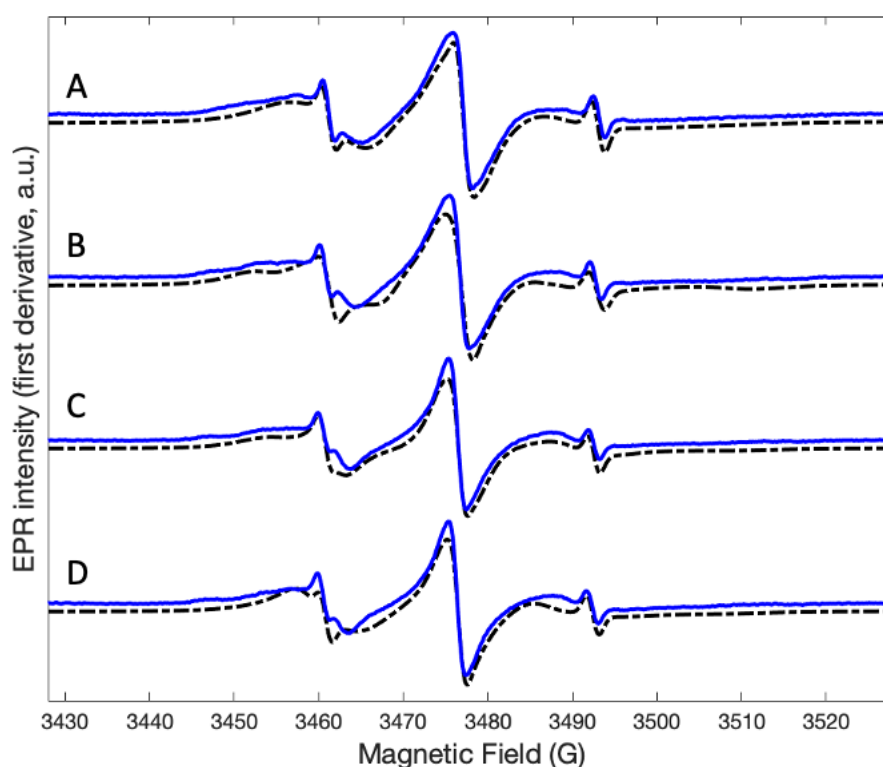


Figure 3.28 RT X-band cw-EPR spectra for Glt_{TK} H334C in detergent micelles (blue) and their respective fits using EasySpin (black dashed line) for the proposed intermediates of the transport cycle: A) apo, B) Na⁺ (50 mM), C) substrate bound (50 mM Na⁺ and 1 mM L-aspartate) and D) inhibitor bound (50 mM Na⁺ and 1 mM DL-TBOA).

	g_{zz}	g_{yy}	g_{xx}	A_{xx} (MHz)	A_{yy} (MHz)	A_{zz} (MHz)	Lwpp (mT)	τ_c (ns)	Ratio
Apo	2.0078	2.0052	2.0016	15.5	14.5	101.4	0.12	3.8	1
	2.0078	2.0052	2.0016	15.5	14.5	101.4	0.15	30.2	0.83
	2.0080	2.0046	2.0024	14.6	13.0	108.0	0.12	0.5	0.06
Na ⁺	2.0078	2.0052	2.0017	14.7	13.9	101.4	0.13	3.8	1
	2.0078	2.0052	2.0017	14.7	13.9	101.4	0.16	7.0	0.84
	2.0080	2.0046	2.0024	14.6	13.0	108.0	0.14	0.5	0.06
Substrate	2.0078	2.0053	2.0017	14.2	13.2	101.4	0.15	3.7	1
	2.0078	2.0053	2.0017	14.2	13.2	101.4	0.13	20.6	0.87
	2.0080	2.0046	2.0024	14.6	13.0	108.0	0.13	0.5	0.05
Inhibitor	2.0078	2.0052	2.0016	14.8	14.1	101.4	0.13	3.8	1
	2.0078	2.0052	2.0016	14.8	14.1	101.4	0.13	8.1	0.93
	2.0080	2.0046	2.0024	14.6	13.0	108.0	0.12	0.5	0.06

Table 3.15 Best fit simulation values for RT X-band cw-EPR spectra of Glt_{TK} H334C in detergent micelles. Results are shown for the four binding states with RMSD values of 0.0240 (apo), 0.0464 (Na⁺), 0.0291 (Na⁺ and L-aspartate), and 0.0354 (Na⁺ and DL-TBOA). The x, y, and x components of the g- and A-tensor and the linewidth (peak to peak, lwpp) are shown for each.

The LT spectra for Glt_{TK} H334C in proteoliposomes are included in Appendix A10.2. The RT spectral simulations for Glt_{TK} H334C in proteoliposomes only required two protein species, as opposed to the additional free-MTSL species present in all other simulations. The results are shown in Figure 3.29 and Table 3.16. These results are similar to those of detergent micelles in both the ratios and τ_c s obtained. In all proteoliposome samples, the most abundant species had the faster rotational correlation time ~ 4.0 ns like in detergent micelles. Apo Glt_{TK} H334C in proteoliposomes had the slowest τ_c of all spectral simulations for this variant at 50.3 ns, which is approximately 20 ns slower than in detergent. This could be due to the presence of the lipid bilayer as it is a relatively exposed residue, perhaps influenced by any protein-lipid interactions. The results for Na⁺ and Na⁺-Inhibitor bound Glt_{TK} H334C were relatively similar between the two environments indicating the same conformational states were present but in slightly different ratios. These simulation observations will be further investigated using pulsed EPR spectroscopy to measure distance distributions between the spin probes.

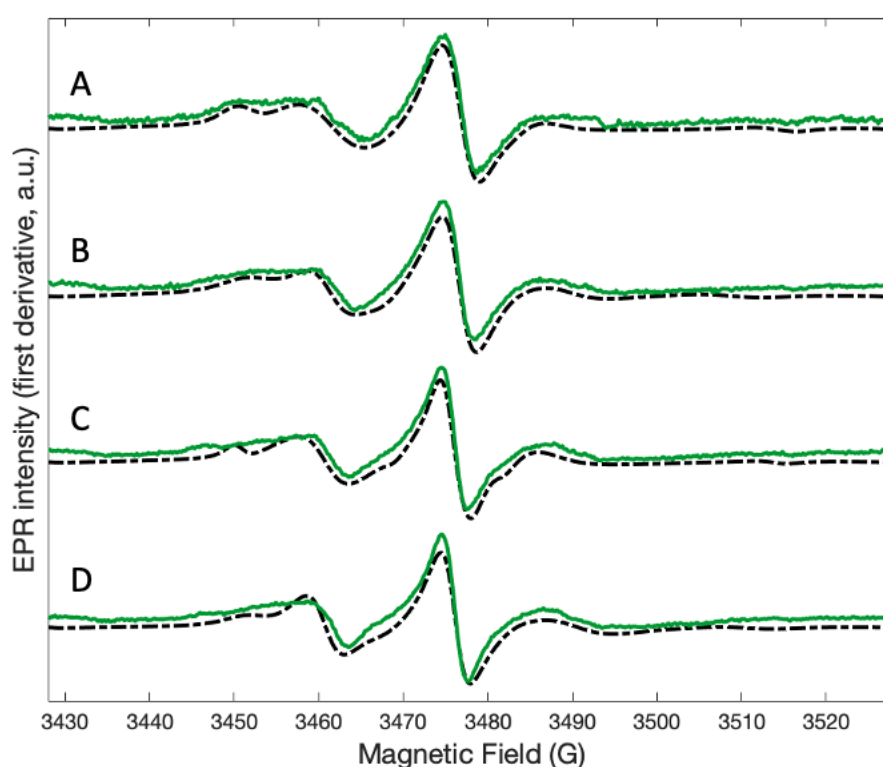


Figure 3.29 RT X-band cw-EPR spectra for Glt_{TK} H334C in proteoliposomes (green) and their respective fits using EasySpin (black dashed line) for the proposed intermediates of the transport cycle: A) apo, B) Na⁺ (50 mM), C) substrate bound (50 mM Na⁺ and 1 mM L-aspartate) and D) inhibitor bound (50 mM Na⁺ and 1 mM DL-TBOA).

	g_{zz}	g_{yy}	g_{xx}	A_{xx} (MHz)	A_{yy} (MHz)	A_{zz} (MHz)	Lwpp (mT)	τ_c (ns)	Ratio
Apo	2.0078	2.0056	2.0020	12.9	13.5	101.4	0.13	4.1	1
	2.0078	2.0049	2.0015	18.5	15.6	101.4	0.18	50.3	0.46
Na ⁺	2.0078	2.0055	2.0021	13.1	15.1	101.4	0.13	2.9	1
	2.0078	2.0056	2.0016	17.2	13.8	101.4	0.18	8.7	0.95
Substrate	2.0078	2.0061	2.0021	12.6	16.7	101.4	0.13	3.6	1
	2.0078	2.0052	2.0015	18.2	11.4	101.4	0.12	18.3	0.25
Inhibitor	2.0078	2.0061	2.0021	12.6	12.1	101.4	0.13	3.8	1
	2.0078	2.0054	2.0015	18.0	14.1	101.4	0.15	13.4	0.51

Table 3.16 Best fit simulation values for RT X-band cw-EPR spectra of Glu_{Tk} H334C in proteoliposomes. Results are shown for the four binding states with RMSD values of 0.0327 (apo), 0.0397 (Na⁺), 0.0389 (Na⁺ and L-aspartate), and 0.0466 (Na⁺ and DL-TBOA). The x, y, and z components of the g- and A-tensor and the linewidth (peak to peak, lwpp) are shown for each.

3.9 Studying the conformational changes of Glt_{Tk} A367C using cw-EPR spectroscopy

3.9.1 Functional studies of Glt_{Tk} A367C in detergent micelles, proteoliposomes and SMALPs using cw-EPR

Glt_{Tk} A367 is located on the functionally important HP2 which is proposed to be a gate to both the intracellular and extracellular environment. The associated dynamics and conformational changes were studied in detergent micelles, proteoliposomes and SMALPs with the addition of substrate or inhibitor, with co-transported Na⁺, to follow the transport cycle. Spectra were recorded using both LT and RT cw-EPR. The RT cw-EPR spectra of Glt_{Tk} A367C in detergent micelles showed clear line shape changes in different binding states, mostly between two different positions highlighted in Figure 3.30. Apo and Na⁺-bound Glt_{Tk} A367C spectral line shapes were very similar with a pronounced peak for spin labelled protein next to that of residual free-MTSL, which shifted position upon addition of substrate and inhibitor. This was the first indication of ion and substrate-induced conformational changes for Glt_{Tk} A367C.

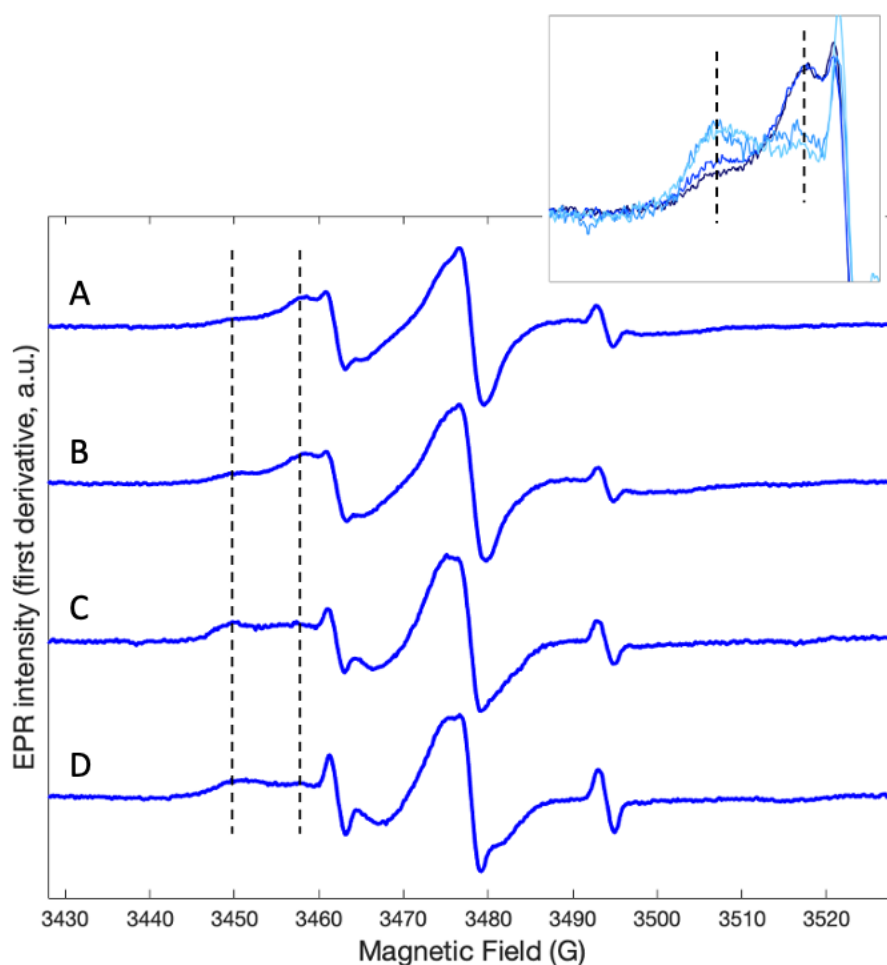


Figure 3.30 X-band RT cw-EPR spectra of Glt_{Tk} A367C in detergent micelles (blue) utilising substrate and co-transported Na⁺ to trap Glt_{Tk} in proposed intermediates of the transport cycle: A) apo, B) Na⁺ (50 mM), C) substrate bound (50 mM Na⁺ and 1 mM L-aspartate) and D) inhibitor bound (50 mM Na⁺ and 1 mM DL-TBOA). Black dashed lines indicate regions of the spectra where subtle changes are observed (see insert). Zoomed in area of the biggest shape changes in the binding conditions shown darkest to lightest for A) to D). Experimental parameters: microwave frequency = 9.758 GHz, centre field = 3478 G, sweep width = 120 G, modulation frequency = 100 kHz, modulation amplitude = 1G, microwave power = 0.1997 mW, number averages = 64.

These functional studies were repeated for Glt_{Tk} A367C reconstituted into proteoliposomes and SMALPs (Figure 3.31). Contrasting to Glt_{Tk} S281C, there were more significant difference in spectral line shapes between the different membrane mimetics. The proteoliposome spectra contained less residual-MTSL with a similar spectral line shape of the spin-labelled protein signal to detergent micelles. SMALPs, however, produced very different results where the spectra for each binding state were similar to each other in the different binding conditions and different to those of detergent micelles and proteoliposomes. There was more free-MTSL present in the

SMALP samples compared to proteoliposomes, further confirming the observations for the other variants that the additional wash steps during liposome reconstitution helps remove residual MTSL. The spectral line shapes of Glt_{Tk} A367C in SMALPs resembled that of Na⁺-inhibitor bound Glt_{Tk} A367C in detergent micelles, with none of the faster τ_c spectral line shape seen for apo and Na⁺ conditions present. These results indicated the spin label experienced a different local environment, which perhaps does not change significantly when in a SMALP under the different binding conditions.

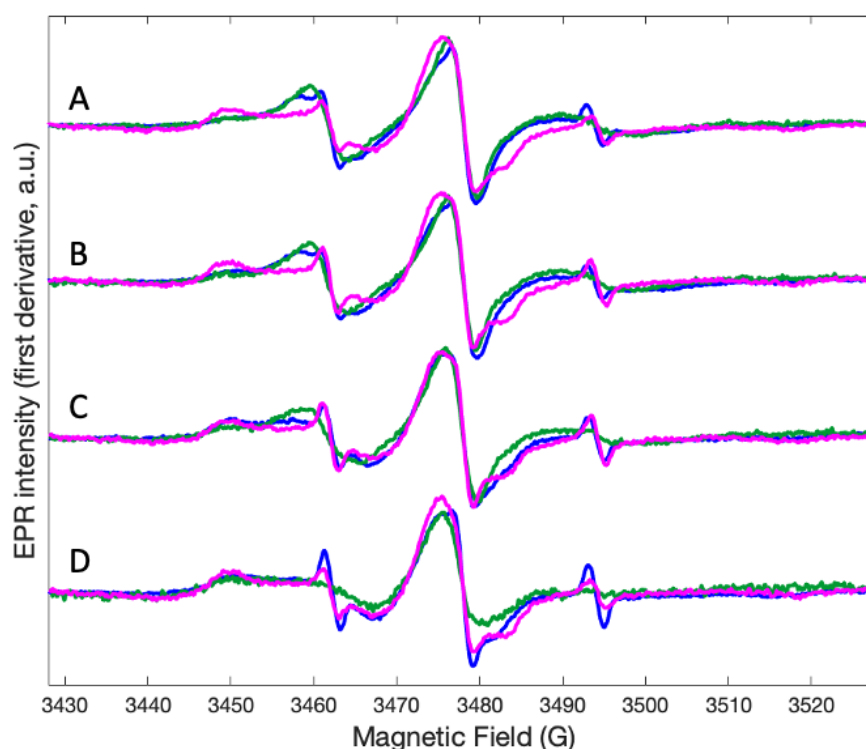


Figure 3.31 X-band RT cw-EPR spectra of Glt_{Tk} A367C in detergent micelles (blue), proteoliposomes (green) and SMALP (pink) utilising substrate and co-transported Na⁺ to trap Glt_{Tk} in proposed intermediates of the transport cycle: A) apo, B) Na⁺ (50 mM), C) substrate bound (50 mM Na⁺ and 1 mM L-aspartate) and D) inhibitor bound (50 mM Na⁺ and 1 mM DL-TBOA). Experimental parameters: microwave frequency = 9.758 GHz, centre field = 3478 G, sweep width = 120 G, modulation frequency = 100 kHz, modulation amplitude = 1–2 G, microwave power = 0.1997 mW, number averages = 64–100.

3.9.2 Spectral simulations of Glt_{Tk} A367C using EasySpin

All LT spectra exhibited very similar line shapes indicating line shape changes in the RT spectra were a result of τ_c . LT cw-EPR spectra were simulated using EasySpin with

the best-fit simulation results for all LT Glt_{TK} A367C spectra included in Appendix A11. The RT simulation results for Glt_{TK} A367C in detergent micelles are shown in Figure 3.32 and Table 3.17. In all four binding states the most abundant species had the fastest τ_c which varied between 3.6–8.1 ns. Based on the number of rotamers from MMM in the different conformations these may correspond to outward facing states. The τ_c values of the two protein species were very similar for the Na⁺ and Na⁺-substrate bound states - particularly for the slower species. This could indicate the conformational change between these two states was minimal compared to the transition between apo and Na⁺ bound states. Inward facing conformations in MMM had much lower numbers of rotamers, hence may correlate to a longer τ_c value. This means apo and Na⁺-inhibitor bound structures with the longer τ_c s (>20 ns) may correspond to an inward facing state. This would align with the Na⁺-inhibitor bound crystal structure. Intermediate outward occluded cryo-EM structures had fewer rotamers than outward open, so the $\tau_c \sim 15$ ns observed for Na⁺ and Na⁺-substrate bound Glt_{TK} A367C is proposed to be an intermediate occluded state.

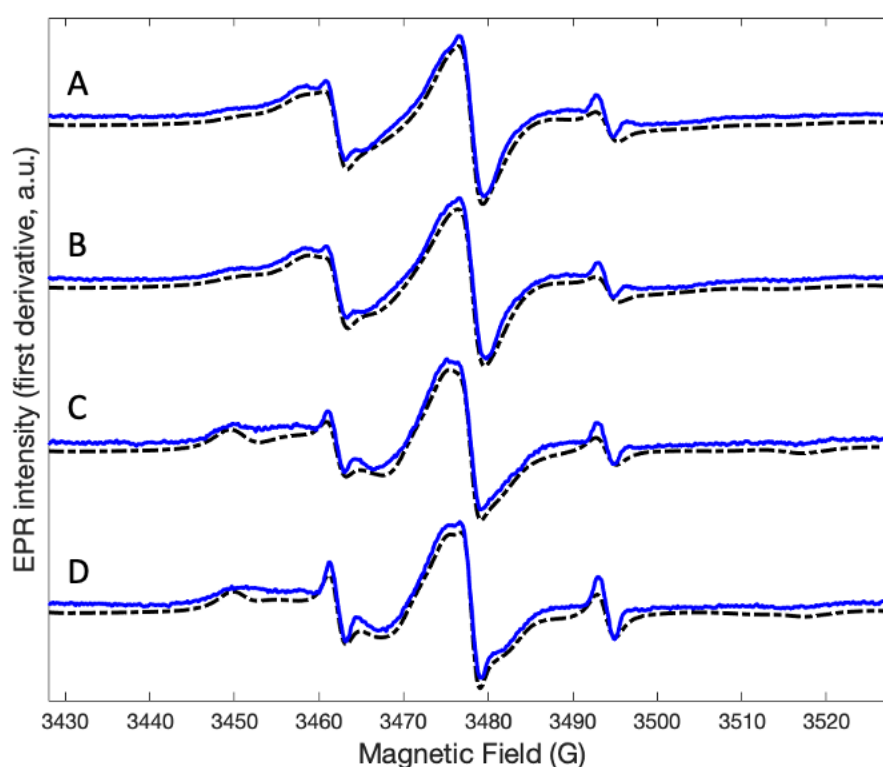


Figure 3.32 RT X-band cw-EPR spectra for Glt_{TK} A367C in detergent micelles (blue) and their respective simulations using EasySpin (black dashed line) for the proposed intermediates of the transport cycle: A) apo, B) Na⁺ (50 mM), C) substrate bound (50 mM Na⁺ and 1 mM L-aspartate) and D) inhibitor bound (50 mM Na⁺ and 1 mM DL-TBOA).

	g_{zz}	g_{yy}	g_{xx}	A_{xx} (MHz)	A_{yy} (MHz)	A_{zz} (MHz)	Lwpp (mT)	τ_c (ns)	Ratio
Apo	2.0076	2.0054	2.0015	13.3	15.1	101.4	0.18	4.4	1
	2.0076	2.0054	2.0015	13.3	15.1	101.4	0.20	27.6	0.23
	2.0077	2.0050	2.0021	8.6	14.6	109.1	0.13	0.6	0.09
Na⁺	2.0076	2.0054	2.0015	15.2	14.7	101.4	0.19	3.6	1
	2.0076	2.0054	2.0015	15.2	14.7	101.4	0.22	14.9	0.32
	2.0077	2.0050	2.0021	8.6	14.6	109.1	0.11	0.6	0.08
Substrate	2.0076	2.0055	2.0014	13.6	15.7	101.4	0.17	8.1	1
	2.0076	2.0055	2.0014	13.6	15.7	101.4	0.22	15.0	0.63
	2.0077	2.0050	2.0021	8.6	14.6	109.1	0.11	0.6	0.10
Inhibitor	2.0076	2.0053	2.0015	12.8	15.6	101.4	0.17	7.2	1
	2.0076	2.0053	2.0015	12.8	15.6	101.4	0.21	22.3	0.68
	2.0077	2.0050	2.0021	8.6	14.6	109.1	0.12	0.6	0.09

Table 3.17 Best fit simulation values for RT X-band cw-EPR spectra of Glt_{rk} A367C in detergent micelles. Results are shown for the four binding states with RMSD values of 0.0256 (apo), 0.0248 (Na⁺), 0.0323 (Na⁺ and L-aspartate), and 0.0338 (Na⁺ and DL-TBOA). The x, y, and x components of the g- and A-tensor and the linewidth (peak to peak, lwpp) are shown for each.

The cw-EPR spectra of Glt_{TK} A367C in proteoliposomes and SMALPs were also analysed using EasySpin and the resulting simulations can be seen in Table 3.18–Table 3.19 and Figure 3.33–Figure 3.34 respectively. Glt_{TK} A367C in proteoliposomes only required two protein species in the simulation routine, without the additional free-MTSL species required for most other samples. All Glt_{TK} A367C simulations in proteoliposomes and SMALPs had the slower τ_c as the least abundant species, as previously observed in detergent micelles. Proteoliposome spectra exhibited similar τ_c s as detergent micelles with the main difference being Na⁺-inhibitor bound where a τ_c of 30.9 ns was extracted compared to 22.3 ns. This could be a result of the lipid bilayer environment and any protein-lipid interactions. When surrounded by native lipids in SMALPs there is a close correlation with the Na⁺-inhibitor bound results in detergent micelles in terms of both ratios of protein species and τ_c s, with very little difference observed between the four binding conditions. The largest difference to detergent micelles was seen for the apo and Na⁺ state where the fastest τ_c observed ~4 ns was not present indicating different conformations were present. Overall, these results suggest for Glt_{TK} A367C reconstitution into SMALPs had a much a larger effect on the local environment of the spin label compared to Glt_{TK} S281C which was investigated further using pulsed EPR for distance measurement as described in Chapter 4.

	g_{zz}	g_{yy}	g_{xx}	A_{xx} (MHz)	A_{yy} (MHz)	A_{zz} (MHz)	Lwpp (mT)	τ_c (ns)	Ratio
Apo	2.0076	2.0054	2.0015	13.3	15.1	101.4	0.18	3.6	1
	2.0076	2.0054	2.0015	13.3	15.1	101.4	0.22	28.7	0.32
Na⁺	2.0076	2.0054	2.0015	15.2	14.7	101.4	0.19	3.6	1
	2.0076	2.0054	2.0015	15.2	14.7	101.4	0.22	16.9	0.24
Substrate	2.0076	2.0055	2.0014	13.6	15.7	101.4	0.17	6.4	1
	2.0076	2.0055	2.0014	13.6	15.7	101.4	0.20	21.3	0.52
Inhibitor	2.0076	2.0053	2.0015	12.8	15.6	101.4	0.19	7.7	1
	2.0076	2.0053	2.0015	12.8	15.6	101.4	0.22	30.8	0.60

Table 3.18 Best fit simulation values for RT X-band cw-EPR spectra of Glt_{TK} A367C in proteoliposomes. Results are shown for the four binding states with RMSD values of 0.0252 (apo), 0.0302 (Na⁺), 0.0276 (Na⁺ and L-aspartate), and 0.0430 (Na⁺ and DL-TBOA). The x, y, and x components of the g- and A-tensor and the linewidth (peak to peak, lwpp) are shown for each.

	g_{zz}	g_{yy}	g_{xx}	A_{xx} (MHz)	A_{yy} (MHz)	A_{zz} (MHz)	Lwpp (mT)	τ_c (ns)	Ratio
Apo	2.0076	2.0054	2.0015	13.3	15.1	101.4	0.17	7.2	1
	2.0076	2.0054	2.0015	13.3	15.1	101.4	0.23	23.1	0.63
	2.0077	2.0050	2.0021	8.6	14.6	109.1	0.12	0.5	0.07
Na⁺	2.0076	2.0054	2.0015	15.2	14.7	101.4	0.20	6.6	1
	2.0076	2.0054	2.0015	15.2	14.7	101.4	0.23	19.5	0.68
	2.0077	2.0050	2.0021	8.6	14.6	109.1	0.12	0.5	0.09
Substrate	2.0076	2.0055	2.0014	13.6	15.7	101.4	0.19	6.0	1
	2.0076	2.0055	2.0014	13.6	15.7	101.4	0.22	24.6	0.66
	2.0077	2.0050	2.0021	8.6	14.6	109.1	0.11	0.5	0.08
Inhibitor	2.0076	2.0053	2.0015	12.8	15.6	101.4	0.18	6.1	1
	2.0076	2.0053	2.0015	12.8	15.6	101.4	0.23	20.7	0.64
	2.0077	2.0050	2.0021	8.6	14.6	109.1	0.11	0.5	0.06

Table 3.19 Best fit simulation values for RT X-band cw-EPR spectra of GlT_{rk} A367C in SMALPs. Results are shown for the four binding states with RMSD values of 0.0255 (apo), 0.0308 (Na⁺), 0.0307 (Na⁺ and L-aspartate), and 0.0280 (Na⁺ and DL-TBOA). The x, y, and x components of the g- and A-tensor and the linewidth (peak to peak, lwpp) are shown for each.

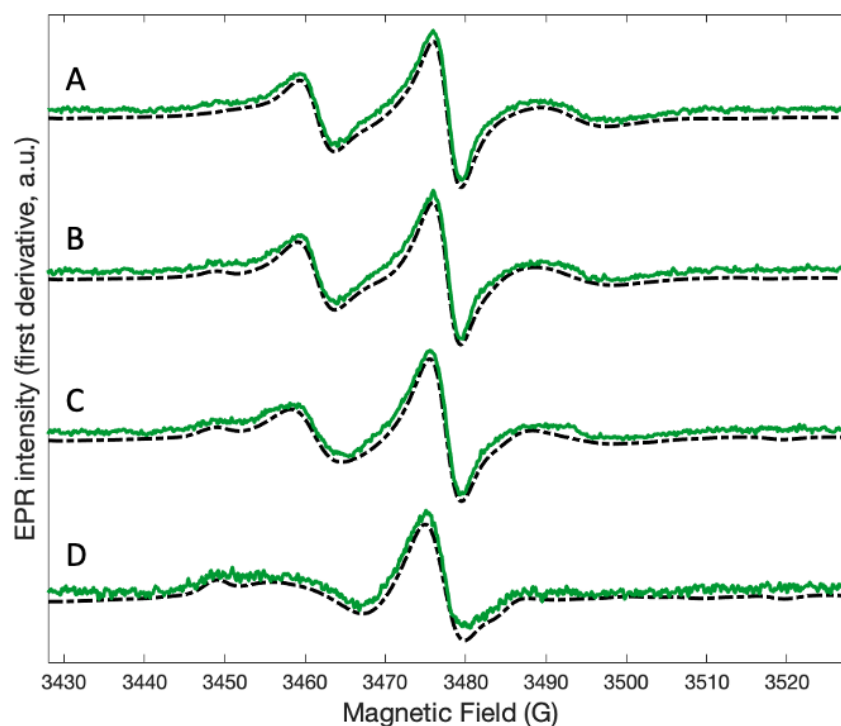


Figure 3.33 RT X-band cw-EPR spectra for Glt_{TK} A367C in proteoliposomes (green) and their respective simulations using EasySpin (black dashed line) for the proposed intermediates of the transport cycle: A) apo, B) Na⁺ (50 mM), C) substrate bound (50 mM Na⁺ and 1 mM L-aspartate) and D) inhibitor bound (50 mM Na⁺ and 1 mM DL-TBOA).

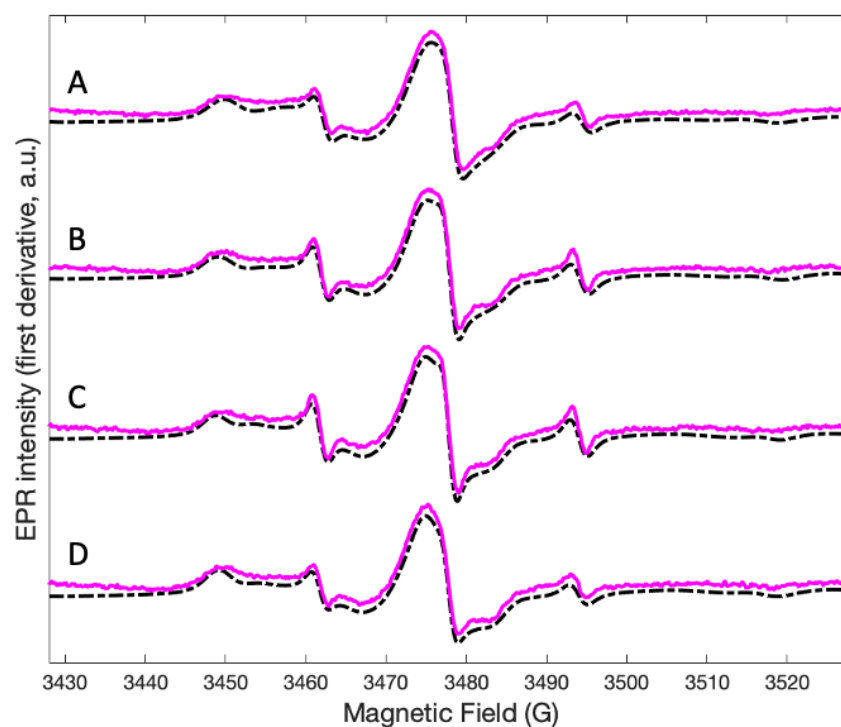


Figure 3.34 RT X-band cw-EPR spectra for Glt_{TK} A367C in SMALP (pink) and their respective simulations using EasySpin (black dashed line) for the proposed intermediates of the transport cycle: A) apo, B) Na⁺ (50 mM), C) substrate bound (50 mM Na⁺ and 1 mM L-aspartate) and D) inhibitor bound (50 mM Na⁺ and 1 mM DL-TBOA).

3.10 Summary of results for Glt_{TK} each binding state

The following tables (Table 3.20–Table 3.23) have been included as a comparison of each Glt_{TK} variant in the four binding conditions. The τ_c values from EasySpin simulations of the two protein species are shown with the relative ratios and the corresponding protein conformations. Glt_{TK} M314C and H334C have not been included as the τ_c values could not be correlated with protein conformations due to the similar number rotamers predicted by MMM. In all four binding states Glt_{TK} is shown to occupy both inward and outward facing conformations, which further supports the proposed alternating access mechanism (Figure 1.17). For different variants, and in some cases membrane mimetics, the same protein conformations are observed but the abundance (i.e. most and least abundant protein species) varies. This suggests there is a small energy difference between the conformations and depending on the position of the spin label, the equilibrium may favour one over the other. If there is a small energy difference between the conformations, when the temperature is reduced to 50 K for distance determination experiments (described in Chapter 4) the relative proportion will change and account for any differences in protein conformation observed between the cw-EPR results and distance determination experiments.

Binding state	Glt _{TK} variant	Membrane mimetic	τ_c (ns)	Ratio	Protein conformations
Apo	S281C	DDM	6.5, 37.9	1:0.32	IF, OFOc
		PL	16.3, 4.8	1:0.89	OF, IF
		SMALP	5.5, 55.7	1:0.60	IF, OFOc
		Nanodisc	2.9, 19.8	1:0.43	IF, OF
	D298C	DDM	2.9, 23.8	1:0.93	IF, OFOc
		PL	6.9, 12.3	1:0.93	iIF, iOFoc
	I312C	DDM	8.7, 51.7	1:0.80	OF, IF
		PL	12.3, 29.2	1:0.57	OFoc, iIFoc
	A367C	DDM	4.4, 27.6	1:0.23	OF, IF
		PL	3.6, 28.7	1:0.32	OF, IF
		SMALP	7.2, 23.1	1:0.63	OFoc, IF

Table 3.20 A summary of EasySpin fitting results for apo Glt_{TK} variants and the corresponding protein conformations: inward facing (IF), outward facing (OF) with additional occluded (Oc) or intermediate (i) suffix and prefixes. Results are included for detergent micelles (DDM), proteoliposomes (PL), SMALPs and nanodiscs.

Binding state	Glt _{TK} variant	Membrane mimetic	τ_c (ns)	Ratio	Protein conformations
Na ⁺	S281C	DDM	17.5, 6.0	1:0.75	OF, IF
		PL	4.5, 21.1	1:0.82	IF, OF
		SMALP	7.1, 50.5	1:0.90	IF, OFOc
		Nanodisc	10.0, 42.2	1:0.91	IF, OFOc
	D298C	DDM	2.8, 12.2	1:0.99	IF, iOFoc
		PL	5.7, 17.7	1:0.73	IF, iOFoc
	I312C	DDM	11.5, 24.1	1:0.76	OFoc, iIFoc
		PL	13.2, 26.3	1:0.76	OFoc, iIFoc
	A367C	DDM	3.6, 14.9	1:0.32	OF, iIFoc
		PL	3.6, 16.9	1:0.24	OF, iIFoc
		SMALP	6.6, 19.5	1:0.68	OFoc, IF

Table 3.21 A summary of EasySpin fitting results for Na⁺ bound Glt_{TK} variants and the corresponding protein conformations: inward facing (IF), outward facing (OF) with additional occluded (Oc) or intermediate (i) suffix and prefixes. Results are included for detergent micelles (DDM), proteoliposomes (PL), SMALPs and nanodiscs.

Binding state	Glt _{TK} variant	Membrane mimetic	τ_c (ns)	Ratio	Protein conformations
Na ⁺ and L-Asp	S281C	DDM	42.3, 9.7	1:0.29	OFOc, IF
		PL	20.5, 3.7	1:0.33	OF, IF
		SMALP	3.6, 46.5	1:0.47	IF, OFOc
		Nanodisc	42.7, 2.3	1:0.41	OFOc, IF
	D298C	DDM	6.3, 11.9	1:0.67	iIF, iOFOc
		PL	5.9, 23.4	1:0.68	iIF, OFOc
	I312C	DDM	12.6, 38.9	1:0.76	OFOc, IFOc
		PL	11.1, 71.4	1:0.99	OFOc, IF
	A367C	DDM	8.1, 15.0	1:0.63	OFOc, iIFOc
		PL	6.4, 21.3	1:0.52	OFOc, IF
		SMALP	6.0, 24.6	1:0.66	OFOc, IF

Table 3.22 A summary of EasySpin fitting results for Na⁺-substrate bound Glt_{TK} variants and the corresponding protein conformations: inward facing (IF), outward facing (OF) with additional occluded (Oc) or intermediate (i) suffix and prefixes. Results are included for detergent micelles (DDM), proteoliposomes (PL), SMALPs and nanodiscs.

Binding state	Glt _{TK} variant	Membrane mimetic	τ_c (ns)	Ratio	Protein conformations
Na ⁺ and DL-TBOA	S281C	DDM	22.9, 7.0	1:0.70	OFO, IF
		PL	3.7, 17.5	1:0.58	IF, OFO
		SMALP	8.4, 23.5	1:0.43	IF, OFO
		Nanodisc	5.8, 45.5	1:0.36	IF, OFOc
	D298C	DDM	3.5, 10.7	1:0.84	IF, iOFOc
		PL	3.9, 13.2	1:0.69	IF, iOFOc
	I312C	DDM	53.8, 8.3	1:0.79	IF, OFO
		PL	55.2, 22.1	1:0.88	IF, iOFOc
	A367C	DDM	7.2, 22.3	1:0.68	OFOc, IF
		PL	7.7, 30.8	1:0.60	OFOc, IF
		SMALP	6.1, 20.7	1:0.64	OFOc, IF

Table 3.23 A summary of EasySpin fitting results for Na⁺-inhibitor bound Glt_{TK} variants and the corresponding protein conformations: inward facing (IF), outward facing (OF) with additional occluded (Oc) or intermediate (i) suffix and prefixes. Results are included for detergent micelles (DDM), proteoliposomes (PL), SMALPs and nanodiscs.

3.11 Studying WT Glt_{Tk} as a control

As a control experiment, wild-type (WT) Glt_{Tk} was incubated with MTSL in an attempt at spin-labelling the Cys-less protein. The same spin-labelling procedure was used as for cysteine variants, followed by recording a cw-EPR spectrum. Due to the absence of native cysteine residues, MTSL cannot form disulfide bonds to attach to the protein at a specific site. However, the cw-EPR spectrum of WT Glt_{Tk} revealed an EPR signal, the shape of which was broader than a residual free-MTSL peak in solution. The sample was subjected to further wash steps using the Zeba™ Spin desalting columns, and the cw-EPR spectrum was re-recorded with no change observed (Figure 3.35). These results indicated the spin label was interacting with WT Glt_{Tk} and required further investigation. The spin labelling efficiency was calculated to be 35% with a protein yield of 0.29 mg/L. The interaction of MTSL with WT Glt_{Tk} is proposed to be a result of non-specific binding and could be used to account for the presence of residual MTSL in the cysteine variants EPR spectra as three sharp peaks. The overall spectral line shape suggested the spin label was in an accessible location where it could undergo fast tumbling in solution such as being stuck to the surface of WT Glt_{Tk}.

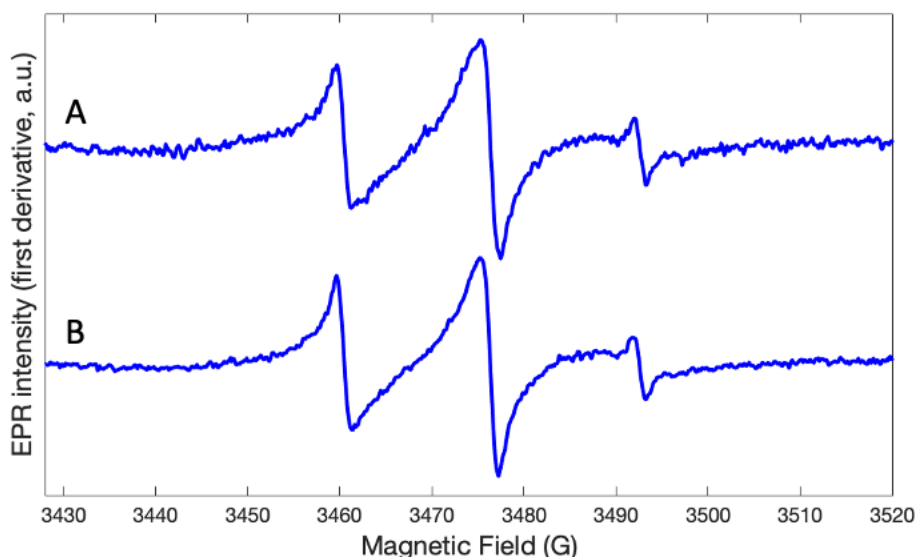


Figure 3.35 X-band RT cw-EPR spectra of apo WT Glt_{Tk} following SDSL after purification (A) and after being subjected to extra wash steps (B). Experimental parameters: microwave frequency = 9.758 GHz, centre field = 3478 G, sweep width = 120 G, modulation frequency = 100 kHz, modulation amplitude = 1G, microwave power = 0.1997 mW, number averages = 64.

The cw-EPR spectrum of spin labelled WT Glt_{TK} was simulated using EasySpin. To effectively replicate the spectrum two MTSL species were required, using typical values for a nitroxide radical as a starting point. The simulation output is shown in Table 3.24 and Figure 3.36. The species with the fastest τ_c only accounted for ~6% of the sample. This species was present at a similar level in the cysteine variant spectra with comparable τ_c values so could be free-MTSL present in all the samples. The other species which accounted for the majority of the spectral line shape was the broader species with a τ_c of 1.5 ns which may be a result of unspecific binding, suggested to be stuck to the hydrophobic surface of the protein. Spin labelling of WT Glt_{TK} and the cysteine variants was conducted using 10 times excess of MTSL per cysteine residue (or protomer protein concentration for WT). Therefore, it is proposed the free-MTSL is significantly depleted in the engineered cysteine variants due to specific binding therefore eliminating the major species observed in the EasySpin simulations of WT.

g_{zz}	g_{yy}	g_{xx}	A_{xx} (MHz)	A_{yy} (MHz)	A_{zz} (MHz)	Lwpp (mT)	τ_c (ns)	Ratio
2.0080	2.0048	2.0025	11.6	19.3	103.7	0.05	1.5	1
2.0078	2.0054	2.0023	14.3	15.7	106.1	0.12	0.5	0.06

Table 3.24 Best fit simulation values for RT X-band cw-EPR spectra of WT Glt_{TK} detergent micelles (RMSD 0.0715).

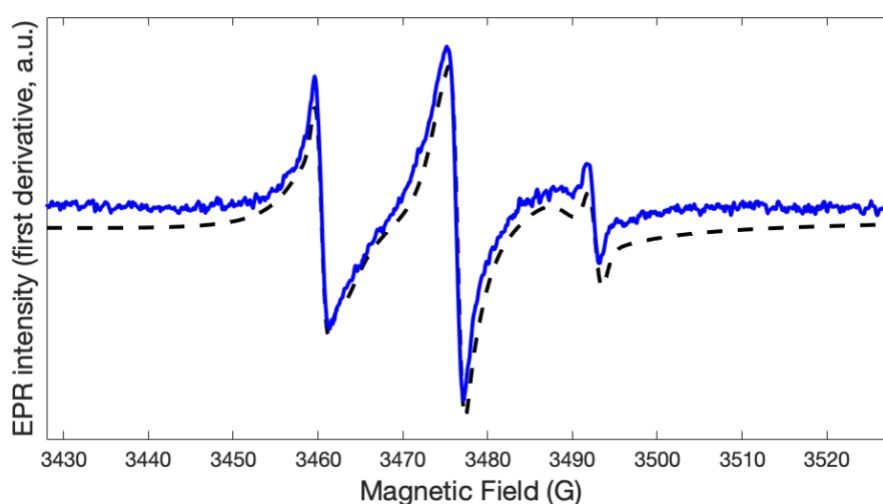


Figure 3.36 RT X-band cw-EPR spectra for WT Glt_{TK} in detergent micelles (blue) and the simulation from EasySpin (black dashed line).

To location of where the MTSL interacted with WT Glt_{TK} is unknown. As with the cysteine variants, WT Glt_{TK} underwent distance determination using pulsed EPR spectroscopy to investigate the presence of dipolar interactions between the spin labels. The raw time decay spectrum obtained from the DEER experiment lacked any exponential decay shape and was unable to be fit using DeerAnalysis software (Figure 3.37). This suggested that there was no dipole-dipole interaction between the MTSL present in the sample, meaning there was no local or organised binding. This further added to the idea of random, unspecific binding on the surface of the protein.

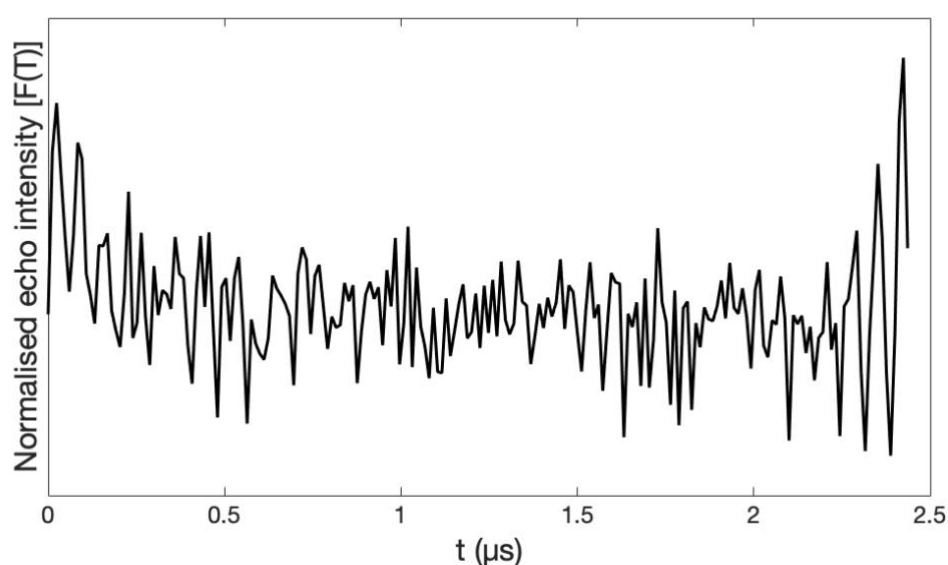


Figure 3.37 The time decay trace of WT Glt_{TK} in a 4-pulse DEER experiment for distance determination.

One alternative method to selecting background functions programmed within DeerAnalysis is to manually subtract a singly labelled background model of the system being studied. This was tested by using the WT Glt_{TK} time trace as a background model to analyse data for the Glt_{TK} S281C Na⁺-aspartate bound sample in detergent micelles. There was no difference in the distance distribution observed which justified both the use of the programmed background models within DeerAnalysis for analysing cysteine variant spectra, as well as confirming this attachment of MTSL observed for WT Glt_{TK} is unlikely to affect the results of the cysteine-variants. It is possible that in the presence of cysteine residues, this unspecific binding does not occur or if it does then it was to a significantly smaller

extent. The free-MTSL signals observed in the cw-EPR spectra of the Glt_{7k} cysteine variants are still thought to be a result of free-MTSL which is supported by the simulation results from EasySpin.

4 Studying the conformational changes of Glt_{Tk} during the transport cycle using pulsed EPR spectroscopy

One of the most common applications of EPR spectroscopy for studying membrane proteins is using pulsed EPR techniques for distance determination. In this thesis the 4-pulse double electron-electron resonance (DEER) method was utilised to study the substrate induced conformational changes of Glt_{Tk} in different environments. To increase the relaxation time of the samples, experiments were carried out at 50 K to maximise the amount of time over which the Hahn echo could be observed (Section 2.3). This in turn allowed determination of longer distances based on dipole-dipole interactions. These results were correlated with the *in silico* predictions using multiscale molecular modelling (MMM)¹⁸³ discussed in Section 3.2 which are based on cryo-EM and crystal structures.

4.1 Initial pulsed EPR optimisation experiments

As discussed in Section 2.3.5, a field-swept echo (FSE) experiment was conducted to determine the optimal pump and observe positions, followed by a T₂ experiment to determine the maximum amount of time the Hahn echo could be observed. A series of experiments were conducted where a 4-pulse DEER experiment was recorded at different pump and observe positions. The modulation depth (λ , Figure 2.18) of the background-corrected DEER spectra was calculated to indicate the number of interacting spins in each experimental set-up (Figure 4.1). Typically, the pump position is selected at the point of maximum EPR intensity in the FSE, with the detect as the second highest point of signal intensity which yielded a modulation depth of 18%. It was found that setting the observe position at the central peak and pumping on the low-field shoulder was optimal for Glt_{Tk} with a modulation depth of 26%, therefore this was applied to all DEER experiments. The lowest modulation depth (8%) was obtained when pumping up-field on the least intense shoulder of the signal and observing at the more intense central position.

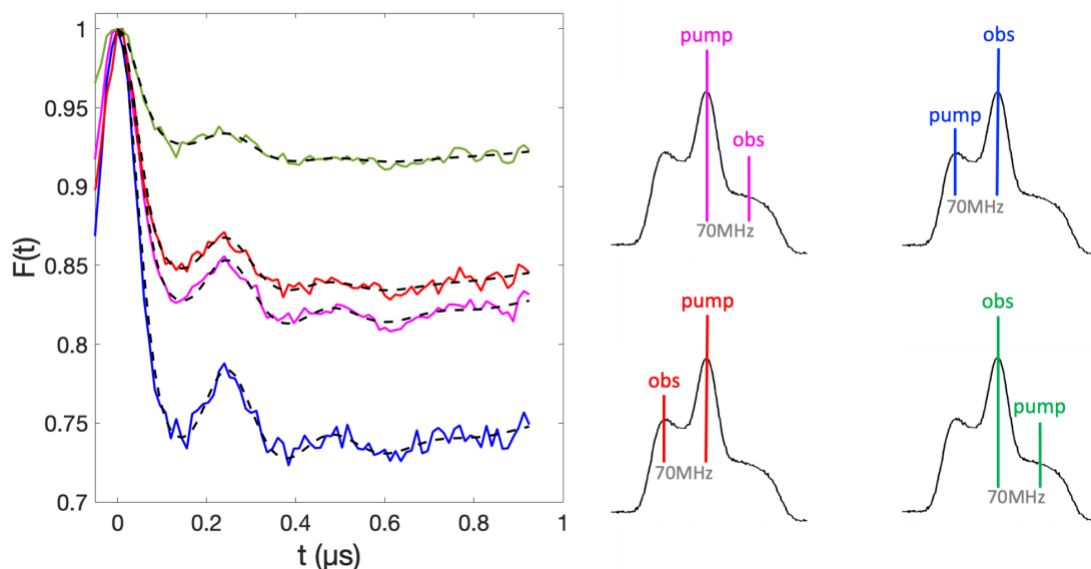


Figure 4.1 Optimisation of the pump and observe positions for DEER experiments of apo Glt_{Tk} S281C. Left: The resultant time decay spectra from different pump and detect positions. Right: the relative positions displayed on the colour coordinated FSE spectra. Experimental parameters are given in Chapter 8.

A second experiment to optimise the frequency offset between the pump and observe position was used. This involved an offset range between 60–90 MHz at 10 MHz intervals, using the optimal pump and observe positions determined above. The modulation depths of each was compared (Figure 4.2). There was little difference between 60 and 70 MHz with modulation depths of 18% and 20% respectively. However, 90 MHz proved to have a significantly reduced signal intensity with a modulation depth of 11%. 60 MHz is the minimum frequency offset that can be used to avoid overlap of the spin probes so 70 MHz was selected for all further experiments.

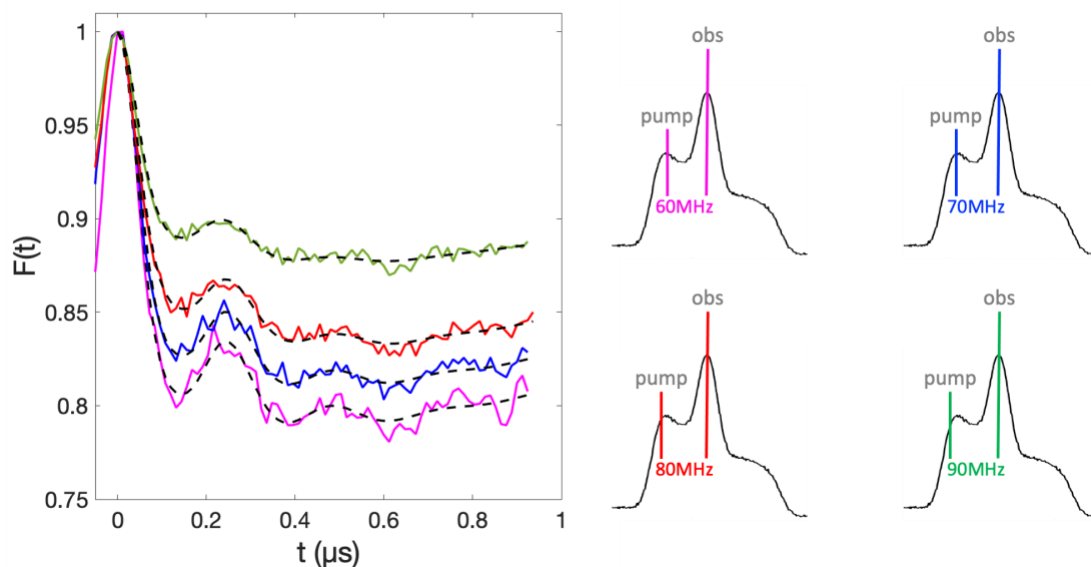


Figure 4.2 Optimisation of the frequency offset for the pump and observe positions for DEER experiments of Glt_{Tk} S281C apo. Left: The resultant time decay spectra from different frequency offsets. Right: the frequency offsets displayed on the colour coordinated FSE spectra. Experimental parameters are given in Chapter 8.

A final experiment was conducted to investigate the relaxation effects upon addition of different viscous solutions, some of which were deuterated. To compare the addition of different viscous solutions, equal volumes of glycerol, d₈-glycerol, and ethylene glycol-d₆ were added to spin-labelled Glt_{Tk} of the same concentration and volume. These additives also function as cryoprotectants to avoid sample damage and create a uniform distribution of protein throughout the sample.¹⁸⁶ The phase memory relaxation time determines the distance distributions which can be determined using DEER studies. Experiments are usually conducted at 50 K which provides a balance between the longitudinal relaxation time (T_1) and the phase memory relaxation time.¹⁶⁶ Without the addition of cryoprotectants the protein, and therefore spin labels, cluster preventing observation of a Hahn echo. This can also happen if not enough is added; 30% is usually the compromise between extending relaxation time and causing rupture of tubes.¹⁸⁶

A primary two-pulse echo sequence (Figure 2.11) was used to determine the phase memory relaxation time, for which spin-spin relaxation (T_2) is a contributor. This experiment is referred to as the T_2 experiment where results are presented in Figure

4.3. It is clear the fastest decay and shortest relaxation time was observed when no glycerol or glycol addition had been made to the sample. The oscillations observed for ethylene glycol- d_6 , and d_8 -glycerol were from the presence of deuterium, which helped to extend the relaxation profile. There was a significant difference in the spectra of samples containing glycerol and d_8 -glycerol as a result of these oscillations. Overall d_8 glycerol achieved the largest extension of phase memory relaxation time, enabling longer times to be used in the DEER set-up and therefore longer distance ranges to be determined. As a result, 30% v/v d_8 -glycerol was added to all detergent solubilised samples, as well as nanodiscs and SMALPs. Proteoliposomes were taken up in deuterium oxide (D_2O) external buffer.

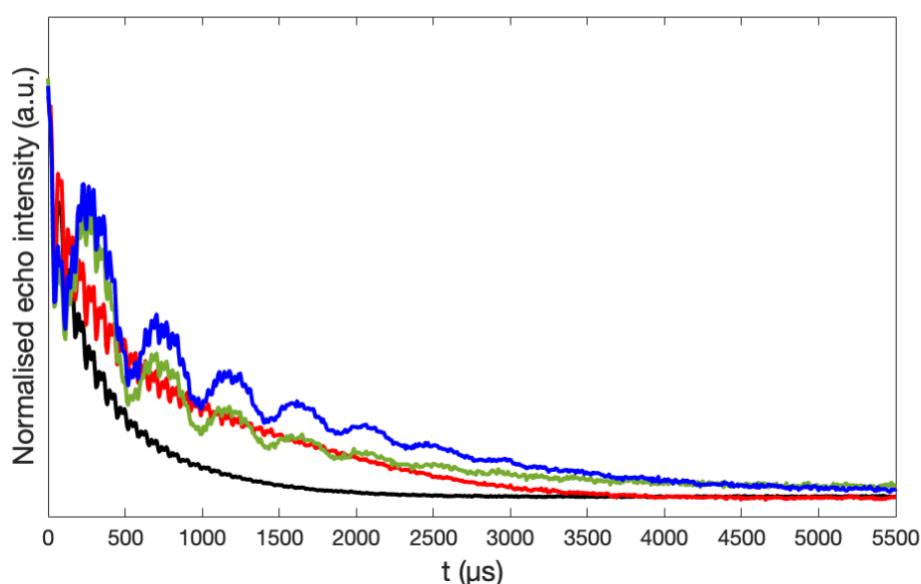


Figure 4.3 The effect of adding different viscous solutions (30% v/v) on the T_2 experiment of apo Glt_{Tk} D298C: no addition (black), glycerol (red), ethylene glycol- d_6 (green), d_8 -glycerol (blue). Experimental parameters are given in Chapter 8.

4.2 Measuring distance changes of Glt_{Tk} S281C using pulsed EPR spectroscopy

4.2.1 Functional studies of Glt_{Tk} S281C in detergent micelles

During the proposed alternating access mechanism, Glt_{Tk} samples a range of conformations including open and occluded for both inward and outward facing states, in addition to intermediate states. The conformations of current Glt_{Tk}

structures are included in Table 3.1 from *in silico* analysis. Glt_{Tk} S281C in detergent micelles was studied using DEER spectroscopy utilising substrate (L-aspartate) inhibitor (DL-TBOA) and co-transported Na⁺ ions to follow the conformational changes at different stages of the transport cycle.

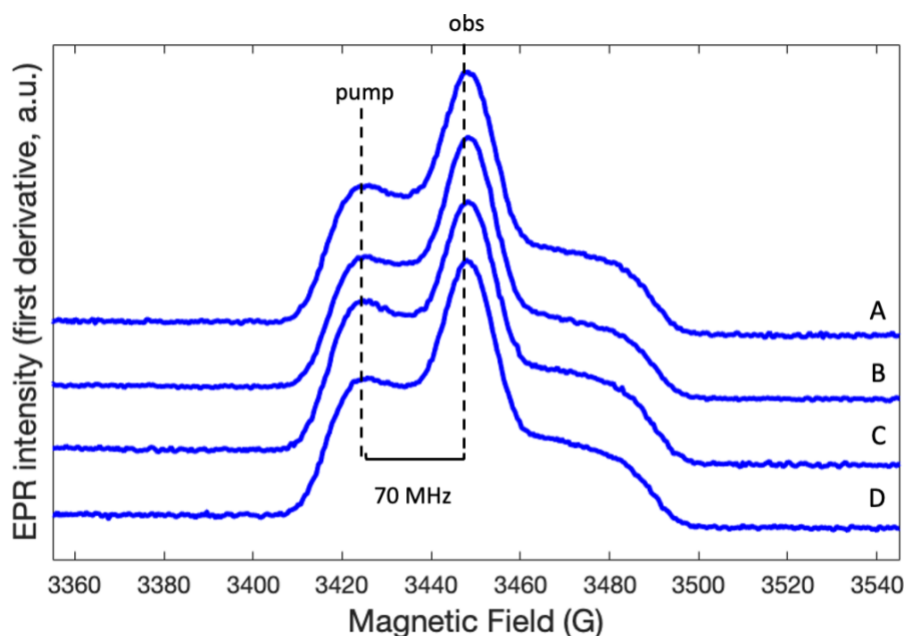


Figure 4.4 X-band field swept echo (FSE) spectra measured for Glt_{Tk} S281C in detergent micelles (50 K). A) apo, B) 50 mM Na⁺, C) 50 mM Na⁺ & 1 mM L-aspartate, D) 50 mM Na⁺ & 1 mM DL-TBOA. Pump and observe positions for DEER experiments are highlighted. Experimental parameters are given in Chapter 8.

Acquisition parameters were optimised as described in the previous section and detailed in Section 8.4.1.5. The FSE spectra are displayed in Figure 4.4 for Glt_{Tk} S281C in each of the four binding conditions, with the pump and detect positions displayed on each. All four spectra were the same, confirming 70 MHz offset between the pump and detect position was suitable for the four samples.

The 4-pulse DEER experiment was used to measure the distance distributions predicted by MMM (Figure 4.5 and discussed previously in Section 3.2). Traces from the DEER experiments were analysed using DeerNet within the DeerAnalysis software to extract distance distributions. The normalised background-corrected time decay spectrum for each sample can be seen in Figure 4.6 alongside the extracted distance

distributions. DeerNet also displays a 95% confidence bound showing the uncertainty in the distance distribution which helped confirm the reliability of the results.

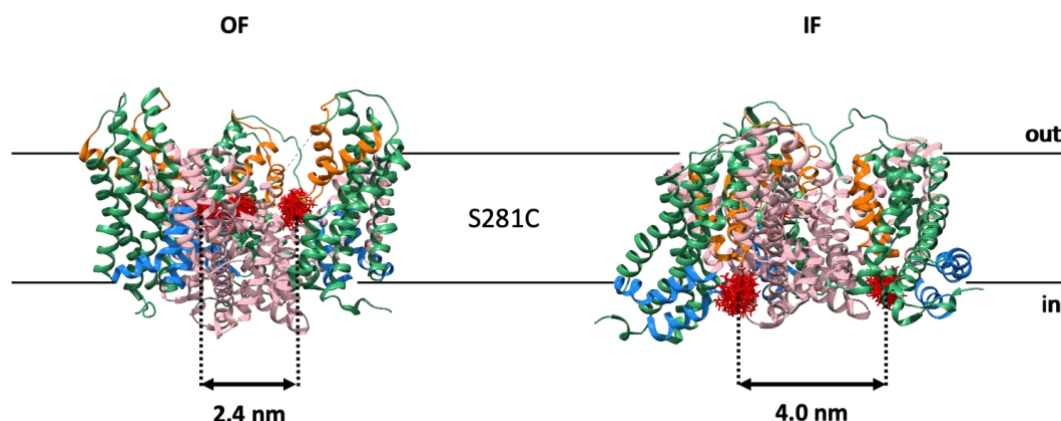


Figure 4.5 MMM results for Glt_{TK} S281C in an outward open (left, PDB 4KY0) and 2 inward open protomers with 1 intermediate outward occluded protomer (right, PDB 6XWR) with MTSL rotamers shown as red clusters. The transport domain (green) and scaffold domain (pink) are shown, with HP1 (blue) and HP2 (yellow) highlighted. The distance distribution is represented by a black arrow, and the distance shown between two protomers in the same conformation.

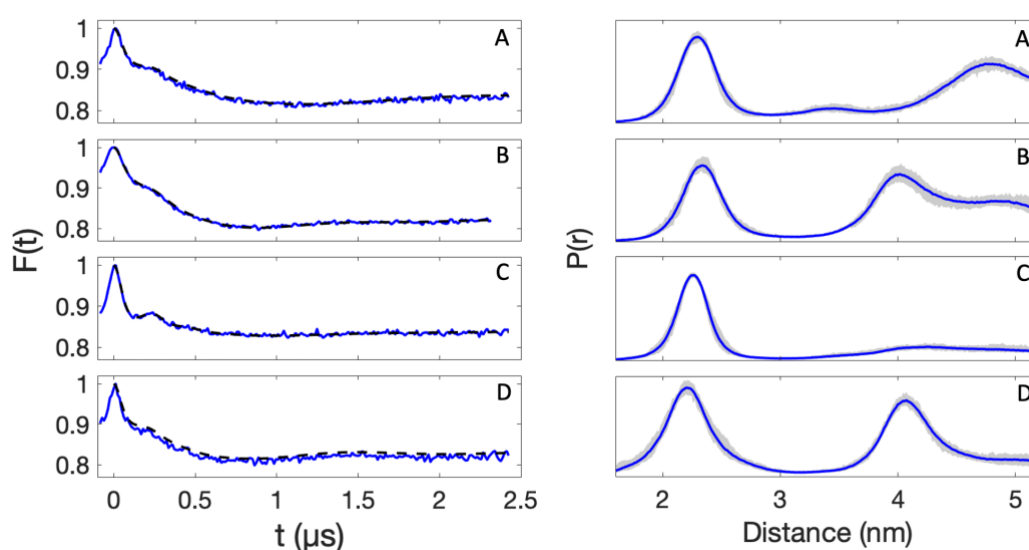


Figure 4.6 4-pulse DEER experiments of Glt_{TK} S281C in detergent micelles utilising substrate and co-transported Na⁺ to trap Glt_{TK} in proposed intermediates of the transport cycle: A) apo, B) Na⁺ (50 mM), C) substrate bound (50 mM Na⁺ and 1 mM L-aspartate) and D) inhibitor bound (50 mM Na⁺ and 1 mM DL-TBOA). The normalised background corrected time decays are shown left with their respective fittings shown as black dashed lines. The right hand traces show the calculated distance distributions using DeerNet and the 95% confidence bounds represented by the light grey areas.

For apo Glt_{TK}, predicted distances of 2.4 nm between S281C on different protomers were calculated based on a cryo-EM structure where each protomer was in an outward facing conformation. The results obtained from DeerNet aligned with these predictions with an additional distance at 5.0 nm. In an apo conformation the protein is likely to be in a variety of conformations including both inward and outward facing states, as seen with the distance distributions. Upon addition and binding of Na⁺ a new distance of 4.0 nm appeared which was likely to represent an inward-open conformation. This was also in agreement with the results from MMM, with the outward facing state (2.4 nm) still present. When substrate (L-aspartate) was added it resulted in a single distance at 2.4 nm, corresponding to the outward occluded state predicted by MMM based on the cryo-EM structure.

The most interesting observation was seen after the addition of inhibitor (DL-TBOA). The cryo-EM structure used for MMM included two inhibitor-bound protomers, and one with just Na⁺. As a result, a mixture of distances was predicted (2.4 nm and 4.0 nm). These results were also observed in the distance distributions confirming it was not an artefact of cryo-EM and indicates that Glt_{TK} protomers operate independently, potentially with functional cooperativity. The potential cooperativity observed was unusual for these systems and has only been previously seen once in WT Glt_{ph} in proteoliposomes.¹³³ The majority of studies on similar systems to Glt_{TK} using techniques such as single molecule fluorescence resonance energy transfer (smFRET) and high speed atomic force microscopy (HS-AFM) show a lack of cooperativity.^{103,104} An alternative explanation for the asymmetry observed in the extracted distances and cryo-EM structure is steric effects where it may not physically have the space to accommodate all three protomers Na⁺-inhibitor bound in an outward facing conformation.

When comparing these results to the simulations of RT spectra using EasySpin (Section 3.4.6), some subtle differences were observed. For example, the simulation results of apo Glt_{TK} S281C required two protein species with the relative ratio of 1:0.32, but visually in Figure 4.6 the ratio looks like it should be closer to 1:0.5. This could be a result of the dynamic state of the protein in solution and the fact DEER is

recorded at 50 K i.e. in a frozen state. Na⁺ and inhibitor bound results had similar ratios of 1:0.70 and 1:0.75 respectively which agreed with the relative intensity of the distance distribution peaks. For substrate bound Glt_{TK}, the EasySpin output was a relative ratio of 1:0.29 but the DEER results showed one very prominent distance. An attempt was made to fit the RT spectra using a single species (as the DEER results suggested) but these were unsuccessful in achieving a close fit. When correlating the results of the distance determination with the EasySpin spectral simulations, there are differences in protein conformations. EasySpin results indicated smaller rotational correlation times (τ_{cs}) aligned with an inward open conformations and larger τ_{cs} corresponded to an outward facing states corresponding to a more restricted environment.

The distance distribution data for Na⁺ contained two different distances which may indicate full saturation had not occurred. As with the cw-EPR studies, the Na⁺-containing samples were repeated with 300 mM Na⁺. Figure 4.7 shows the time decay traces plus fits of the data used to predict distance distributions shown in the right hand panel at both 50 mM and 300 mM Na⁺. There was very little difference in the results obtained, particularly for the substrate bound state. Both Na⁺ and inhibitor bound conditions still contained two distance distributions and therefore two conformational states of Glt_{TK} S281C. These experiments further confirmed that 50 mM Na⁺ was enough to achieve saturating conditions in these EPR studies.

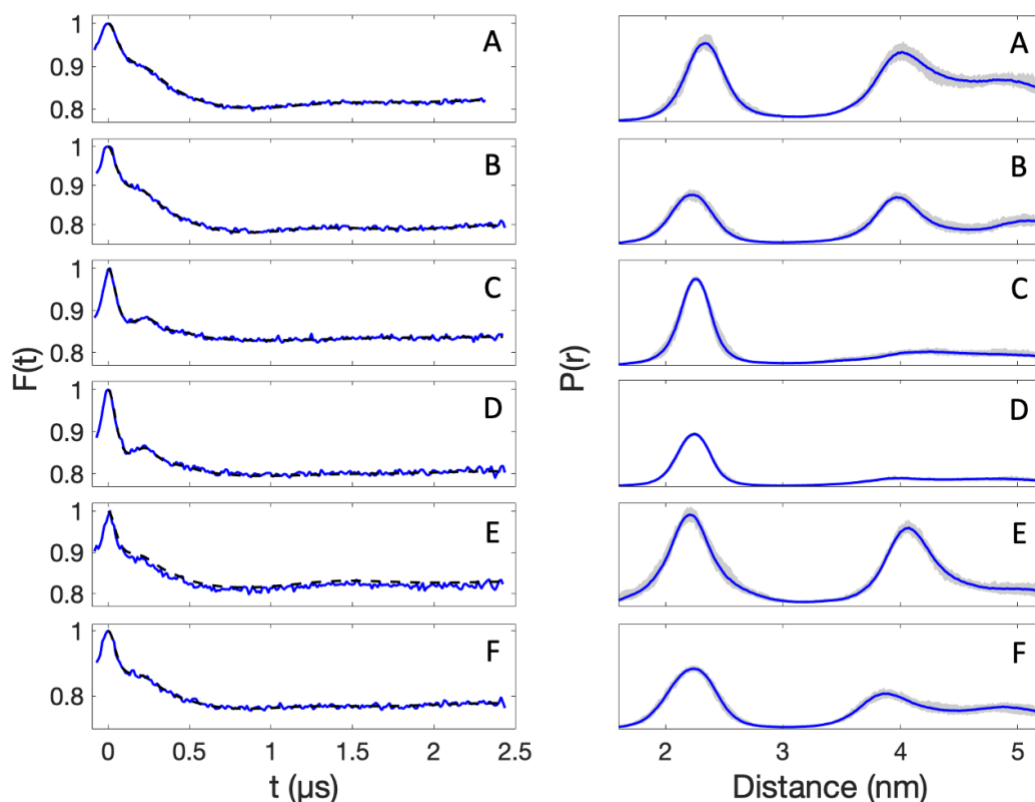


Figure 4.7 4-pulse DEER experiments of Glt_{TK} S281C in detergent micelles at 50 mM Na⁺ compared to 300 mM Na⁺. The different binding states recorded were A) 50 mM Na⁺, B) 300 mM Na⁺, C) substrate bound (50 mM Na⁺ & 1 mM L-aspartate), D) substrate bound (300 mM Na⁺ & 1 mM L-aspartate), E) inhibitor bound (50 mM Na⁺ & 1 mM DL-TBOA), and F) inhibitor bound (300 mM Na⁺ & 1 mM DL-TBOA). The normalised background corrected time decays are shown left with their respective fittings shown as black dashed lines. The right hand traces show the calculated distance distributions using DeerNet and the 95% confidence bounds represented by the light grey areas.

Studies of Glt_{ph}, which has a 77% sequence identity to Glt_{TK}, showed successful binding of L-glutamate as well as the preferential substrate L-aspartate.¹¹⁰ However, to date no data for Glt_{TK} with L-glutamate has been published. 4-pulse DEER experiment was performed on Glt_{TK} S281C with 1 mM L-glutamate (and 50 mM Na⁺) to investigate whether the same result with L-aspartate was observed. However, a mixture of distances were observed like the Na⁺ bound state, with the biggest peak being the longer distance of 4.0 nm. A second study was conducted where an excess of L-aspartate was then added to the same sample to see whether it displaced any L-glutamate, resulting in the single distance observed for the aspartate-bound sample. However, a mixture of conformations remained (Figure 4.8), further adding

to the uncertainty of the nature of the interaction between Glt_{7k} and L-glutamate also found in STD-NMR experiments (Section 6.1).

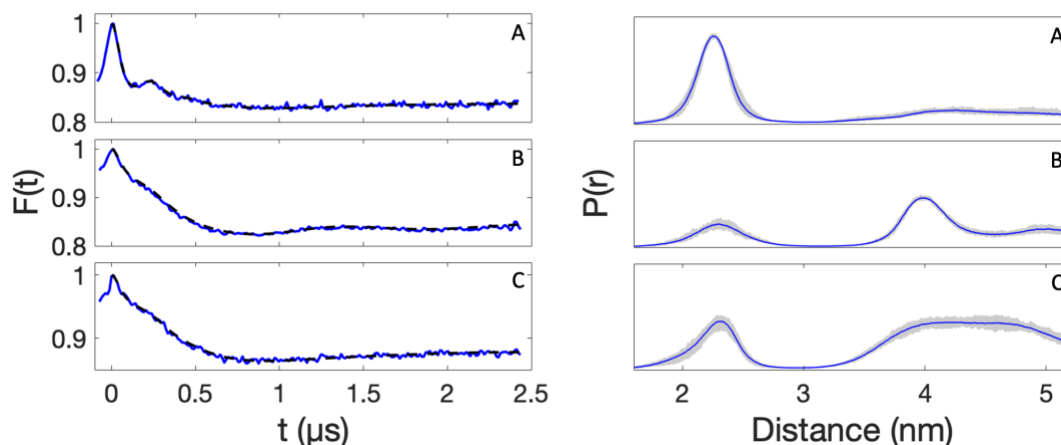


Figure 4.8 4-pulse DEER experiments of Glt_{7k} S281C in detergent micelles with 50 mM Na⁺ and additions of A) 1 mM L-aspartate, B) 1 mM L-glutamate and C) 1 mM L-glutamate followed by 1 mM L-aspartate. The normalised background corrected time decays are shown left with their respective fittings shown as black dashed lines. The right hand traces show the calculated distance distributions using DeerNet and the 95% confidence bounds represented by the light grey areas.

Overall, these results indicated the presence of a binding interaction between Glt_{7k} and L-glutamate, otherwise addition of L-aspartate would be expected to give the single distance distribution observed previously (shown in Figure 4.8). The distance distributions present for L-glutamate with Glt_{7k} S281C indicate the presence of inward facing conformations, in addition to the outward occluded conformation at 2.3 nm seen with L-aspartate. Due to the alternating access mechanism, it is possible to have substrate bound protein in both outward and inward facing conformations. The protein binds substrate in outward facing conformation and transitions through intermediate states before being release in an inward facing conformation. Therefore, the distance of 4 nm could correspond to inward facing, substrate bound Glt_{7k} which has not been observed using structural techniques. Structurally similar Glt_{ph} has been solved in an inward-facing state bound to L-aspartate and co-transported Na⁺.¹²¹

4.2.2 Functional studies of Glt_{7k} S281C in more native environments

Equivalent experiments to those described in Section 4.2.1 were on Glt_{7k} S281C reconstituted into proteoliposomes, nanodiscs and purified into SMALPs. These samples were also studied using DEER experiments in the four binding states, and data was subsequently analysed using DeerNet. Table 4.1 summarises the distance distributions obtained for each sample, including detergent micelles. The time decay traces plus fits of the data used to predict distance distributions are shown in Figure 4.9. Lower protein concentration obtained for some of the environments truncated the time window over which the Hahn echo (τ_2) was observable resulting in a shorter maximum detectable distance between nitroxide spin probes (as shown for proteoliposomes and SMALPs in Figure 4.9).

	Distance distribution obtained (nm)			
	Detergent	Proteoliposomes	SMALP	Nanodiscs
Apo	2.4, 5.0	2.4, 4.1	2.5, 4.0, 5.0	2.4, 4.4
Na ⁺	2.4, 4.0	2.4, 4.1	2.4, 4.0	2.4, 4.4
substrate	2.4	2.4	2.5, 3.5, 4.0	2.4
Inhibitor	2.4, 4.0	2.4*, 4.1	2.4, 4.0	2.3, 4.2

Table 4.1 A summary of the distance distributions calculated (using DeerNet) from the 4-pulse DEER traces of Glt_{7k} S281C. Studies were conducted in four different protein environments, in all four binding states using 50 mM Na⁺, 1 mM L-aspartate and 1 mM DL-TBOA. *Means this distance is covered within the uncertainty region so may not be real.

The results in Table 4.1 and Figure 4.9 showed clear differences in the distance distributions between the four environments. Generally, the apo- and Na⁺ bound states presented similar results for all protein environments, with the only differences being a result of the different τ_2 used or in the fractional intensities of the peaks which represented different population states. SMALP and nanodiscs were the most similar environment to each other with the main difference being the presence of native lipids in SMALPs. Nanodiscs and liposomes showed very similar results, the main difference being the fractional intensities of the peaks for inhibitor bound Glt_{7k} S281C.

In proteoliposomes, the Na⁺-TBOA peak at 2.4 nm was the same height as the uncertainty distribution so it cannot be said with 95% confidence that this distance exists. However, the unusual asymmetric behaviour seen in detergent micelles was also present in the other inhibitor bound structures. To check whether this was incomplete binding or due to the protein environment, more DL-TBOA was added which resulted in no change in distance distribution suggesting binding was complete.

When comparing the distances for inhibitor bound Glt_{TK} S281C in proteoliposomes to MMM predictions, the protein was likely to be in an inward facing conformation where all three distances were the same (4.0 nm), or a mixture of conformations were present resulting in a distance of 2.4 nm between spins in the sample ensemble. Given that DL-TBOA is a competitive inhibitor, preventing closure of the extracellular gate the latter argument was more likely. In Glt_{ph}, the inhibitor bound structure is often observed in an outward facing conformation so a proportion of the protomers are likely to be in this state due to the nature of the inhibition, particularly since little change was observed on further addition of DL-TBOA.

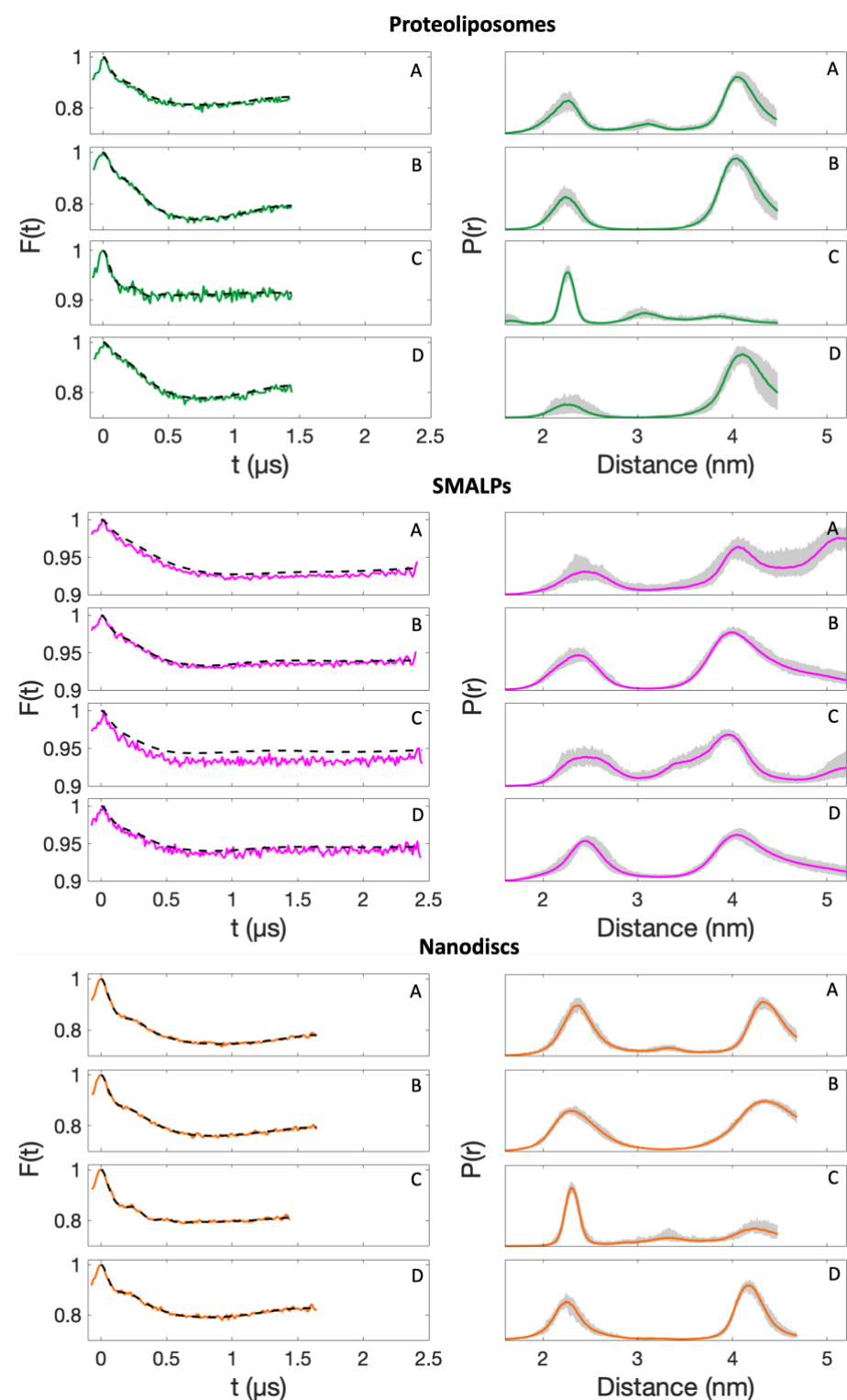


Figure 4.9 4-pulse DEER experiments of Glt_{Tk} S281C in proteoliposomes (green), SMALPs (pink) and nanodiscs (orange) utilising substrate and co-transported Na⁺ to trap Glt_{Tk} in proposed intermediates of the transport cycle: A) apo, B) Na⁺ (50 mM), C) substrate bound (50 mM Na⁺ and 1 mM L-aspartate) and D) inhibitor bound (50 mM Na⁺ and 1 mM DL-TBOA). The normalised background corrected time decays are shown left with their respective fittings shown as black dashed lines. The right hand traces show the calculated distance distributions using DeerNet and the 95% confidence bounds represented by the light grey areas.

The other noteworthy result from this set of data was for Glt_{Tk} S281C in SMALP. This was the only substrate bound sample of Glt_{Tk} S281C which did not produce a single distance at 2.4 nm, even upon addition of extra substrate. The initial addition of 1 mM L-aspartate resulted in a new peak at 3.5 nm. When excess substrate was added, this shifted to be 3 nm (Appendix A12). For the intermediate outward-occluded (substrate bound) state MMM predicted a distance of 3.0 nm, as seen in the presence of excess substrate. The 3.5 nm distance was proposed to be for an intermediate inward-occluded state, as the inward facing conformations have longer distance distributions, and the distance is similar to that of the outward occluded conformation. There was a peak at 4.0 nm in all samples for Glt_{Tk} S281C in SMALPs, which for all the other environments disappeared when substrate was added. This raised the question of whether all three protomers successfully bound substrate when encapsulated in a SMALP and could be a point of further investigation in the future using binding and transport assays. In the proposed alternating access mechanism, Glt_{Tk} binds substrate in an outward facing conformation and transitions via intermediates to an inward facing conformation where the substrate is released to the intracellular environment. Therefore, these results could indicate in a SMALP environment there is greater conformational flexibility which has led to new conformational states observed when substrate bound.

4.3 Measuring distance changes of Glt_{Tk} D298C using pulsed EPR spectroscopy

The 4-pulse DEER method was then applied to Glt_{Tk} D298C in detergent micelles and proteoliposomes for distance determination. The protein was studied in the four different binding states to follow the binding induced conformational changes. Glt_{Tk} D298 was in the most exposed site to the intracellular environment, so revealed information about movements on this side of the membrane. Distance distributions extracted from the data using DeerNet (within DeerAnalysis) were compared to those predicted by MMM. Results from the 4-pulse DEER experiment of Glt_{Tk} D298C in detergent micelles are shown in Figure 4.10.

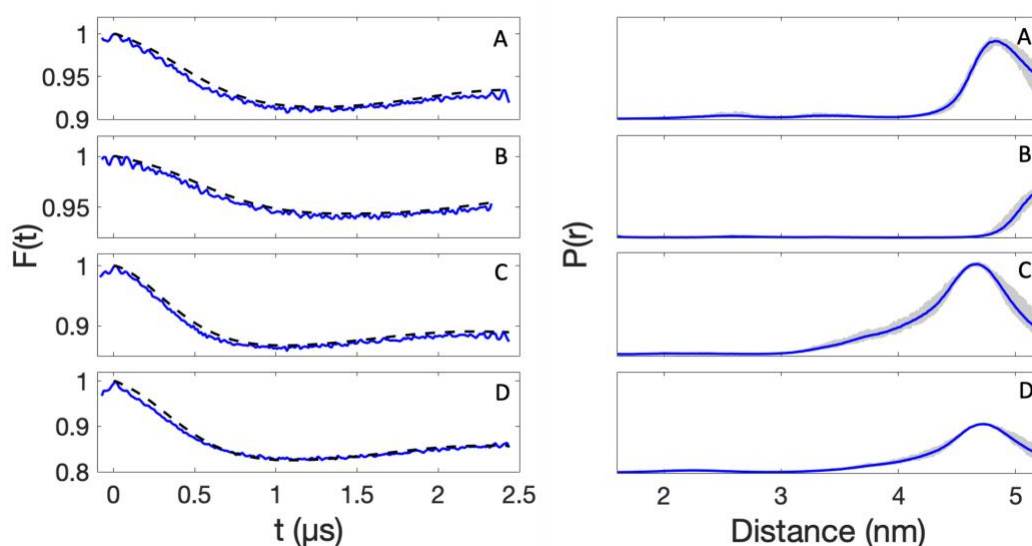


Figure 4.10 4-pulse DEER experiments of Glt_{Tk} D298C in detergent micelles utilising substrate and co-transported Na⁺ to trap Glt_{Tk} in proposed intermediates of the transport cycle: A) apo, B) Na⁺ (50 mM), C) substrate bound (50 mM Na⁺ and 1 mM L-aspartate) and D) inhibitor bound (50 mM Na⁺ and 1 mM DL-TBOA). The normalised background corrected time decays are shown left with their respective fittings shown as black dashed lines. The right hand traces show the calculated distance distributions using DeerNet and the 95% confidence bounds represented by the light grey areas.

The predicted distance distributions using MMM (Section 3.1 and Figure 4.11) based on cryo-EM and crystal structures range between 3.6–6.8 nm. Using a τ_2 of 2.5 μs meant that only distance distributions of up to 5.0 nm could be observed, therefore limiting the results that could be obtained (Figure 4.10). This is one of the obstacles of studying membrane proteins using techniques which require high protein concentrations and volume (ideally >70 μM and 70–80 μL of protein for DEER experiments at X-band) even with the addition of d₈ glycerol to help extend the relaxation time. The similar line shape observed for Glt_{Tk} D298C at RT X-band was reflected in the very similar distance distributions calculated using DeerNet. These results also corresponded to the similar τ_c values obtained using EasySpin indicating environment of D298C is similar in all states of the protein, contrasting to Glt_{Tk} S281C.

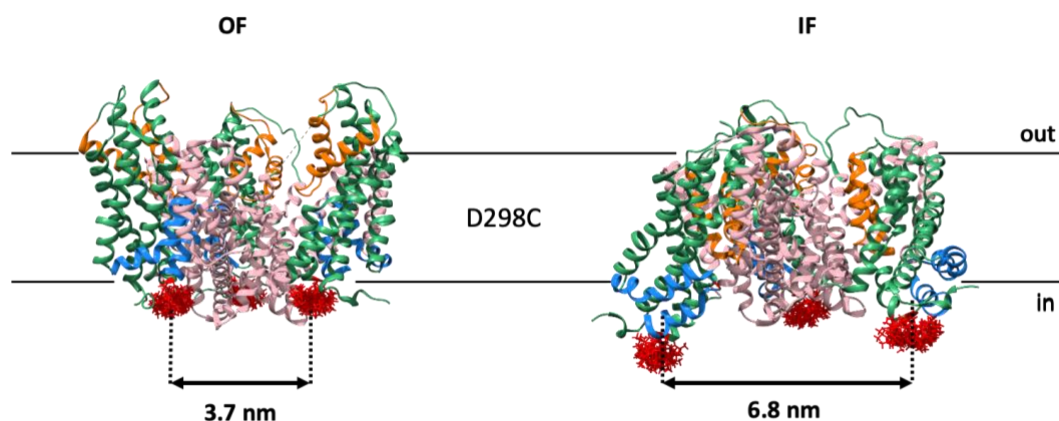


Figure 4.11 MMM results for Glt_{Tk} D298C in an outward open (left, PDB 4KY0) and 2 inward open protomers with 1 intermediate outward occluded protomer (right, PDB 6XWR) with MTSL rotamers shown as red clusters. The transport domain (green) and scaffold domain (pink) are shown, with HP1 (blue) and HP2 (yellow) highlighted. The distance distribution is represented by a black arrow, and the distance shown between two protomers in the same conformation.

DeerNet calculated a distance distribution of 4.7 nm for Glt_{Tk} D298C in an apo state, with a similar distance of 4.6 nm observed for both the substrate and inhibitor bound states which was likely to be an outward facing conformation. The MMM results showed the shorter distance distributions corresponded to protomers in outward facing states, with the longer distances representative of inward-facing states. The closest distance to 4.6 nm from MMM was for an intermediate outward occluded conformation when substrate-bound with a distance of 4.4 nm. TBOA is a competitive inhibitor, so it was unsurprising a similar result had been obtained for a substrate bound structure. It locks the protein in an outward facing state by preventing closure of the extracellular gate, which corresponded to the results obtained for Glt_{Tk} D298C. It was clear from Figure 4.10 that the Na⁺ bound state had a longer distance distribution (>5.0 nm) which had been cut off. It was possible, if the sample quality had allowed, this could correspond to the predicted MMM distance of 5.5–6.8 nm for this state. Inward facing conformations could be present in Glt_{Tk} D298C, showing the asymmetry observed in Glt_{Tk} 281C, but the distance distributions corresponding to this were bigger than could be achieved experimentally. The concentration of the sample was too low, and relaxation was too rapid so reliable conclusions could not be made about conformational states.

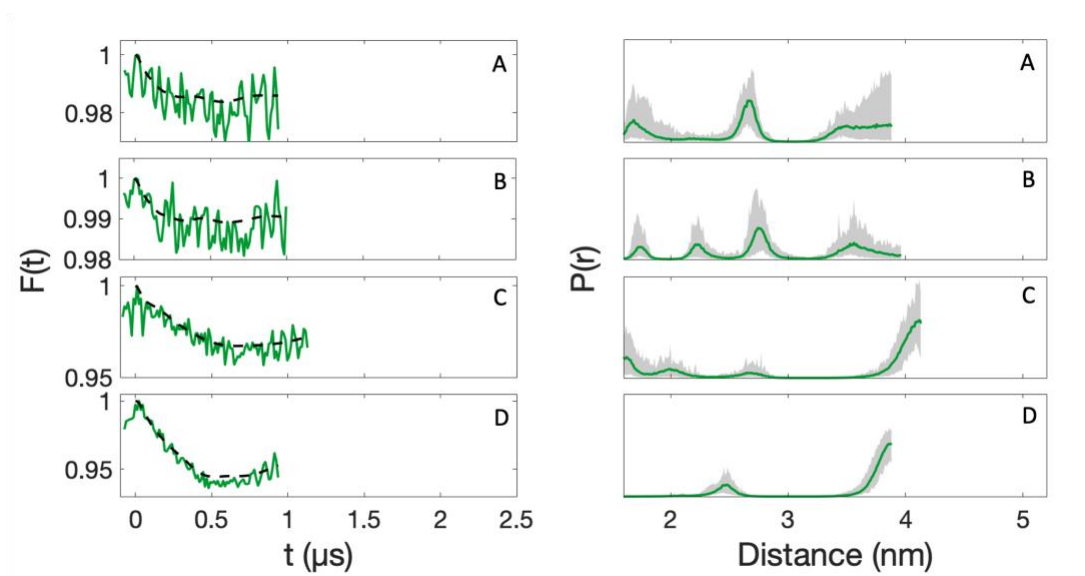


Figure 4.12 4-pulse DEER experiments of Glt_{Tk} D298C in proteoliposomes utilising substrate and co-transported Na⁺ to trap Glt_{Tk} in proposed intermediates of the transport cycle: A) apo, B) Na⁺ (50 mM), C) substrate bound (50 mM Na⁺ and 1 mM L-aspartate) and D) inhibitor bound (50 mM Na⁺ and 1 mM DL-TBOA). The normalised background corrected time decays are shown left with their respective fittings shown as black dashed lines. The right hand traces show the calculated distance distributions using DeerNet and the 95% confidence bounds represented by the light grey areas.

The binding induced conformational changes of Glt_{Tk} D298C in proteoliposomes was also investigated using DEER experiments. However, reaching the longer distance distributions predicted for this variant was difficult; when combined with the additional challenge of achieving a comparable protein concentration in proteoliposomes it resulted in very noisy traces (Figure 4.12). Due to the low sample concentration of Glt_{Tk} D298C in proteoliposomes, a τ_2 of only 1 μs was used allowing distances of up to ~4.0 nm to be predicted. This was just above the shortest distance predicted by MMM. The distances measured for Glt_{Tk} D298C in detergent micelles were all greater than 4.6 nm. Therefore, it was unsurprising the results obtained did not lead to any reliable distance distributions. When looking at the experimental time decays and their fits in Figure 4.12 it was clear the fits have artificial oscillations introduced due to the noise of the traces. This resulted in falsely predicted distance distributions with high levels of uncertainty. For the apo and Na⁺ states almost the entire region underneath the predicted distances was uncertain and they did not make physiological sense based on predicted distances from published structures.

The substrate and inhibitor-bound time decays were less noisy, and therefore had a better fit, but the predicted distance distributions were cut off. Although if taken as the cut-off point at ~4.0 nm they may have corresponded to an outward facing or outward occluded conformation based on MMM. This may suggest that Na⁺-substrate and Na⁺-inhibitor bound Glt_{TK} D298C in proteoliposomes has a more compact structure than detergent micelles due to the start of the peak at shorter distance distributions.

4.4 Measuring distance changes of Glt_{TK} I312C using pulsed EPR spectroscopy

Pulsed EPR spectroscopy was used to further to investigate Glt_{TK} I312C in detergent micelles and proteoliposomes, focusing on any conformational changes in the different binding states. At RT X-band the cw-EPR spectra showed subtle changes in line shape reflected in the EasySpin results which may correspond to different conformational states. To extend the lifetime of the signal (and phase memory relaxation time) d₈-glycerol was added to the samples, however the lifetime of the decay stayed the same (Figure 4.13).

Deuteration was expected to introduce oscillations into the spectra, as shown in Figure 4.3 previously. This behaviour indicated that the spin label bound to I312C was not accessible (or solvent exposed) which made a big difference to the glycerol effects; this was seen for all four binding states. Glt_{TK} I312C was very close to the highly conserved NMDGT motif which is involved in Na⁺ and substrate binding. This may account for the inaccessibility observed. To ensure the stock of d₈ glycerol was not the cause of the lack of oscillations, a new sample was prepared using a new bottle of d₈ glycerol giving the same results. An even larger excess of d₈ glycerol was added, again with the same results. These results meant the distance distributions determined in the DEER experiment were restricted.

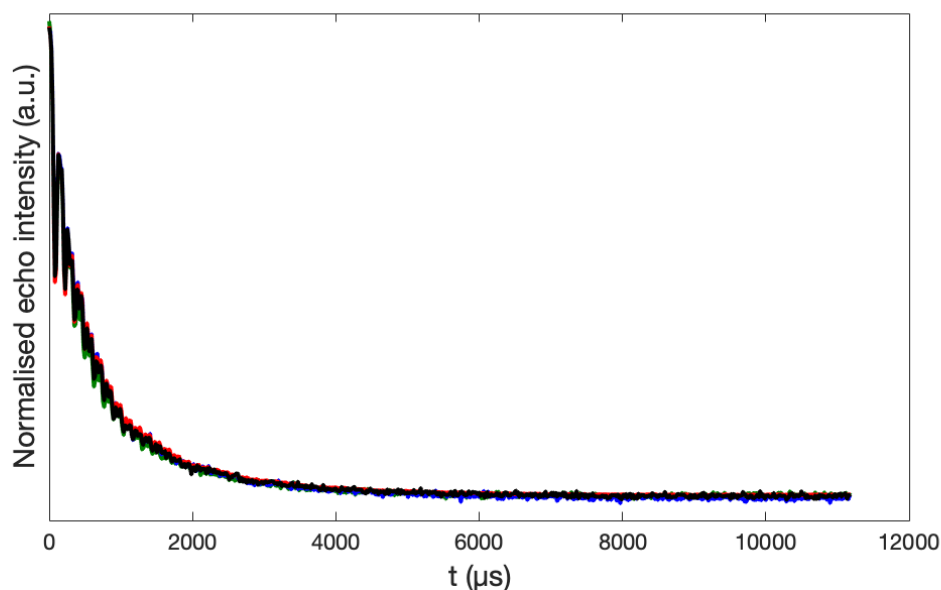


Figure 4.13 The effects of glycerol on the T_2 experiment of Glt_{Tk} I312C in an apo state. The spectra shown represent no glycerol present (black), after the addition of 30% v/v d₈-glycerol (blue), the addition of 30% v/v new d₈-glycerol (red) and a further excess of new d₈-glycerol (green).

Despite d₈ glycerol having no effect on the relaxation of the spins, it was added to all Glt_{Tk} I312C detergent samples for distance distribution experiments for consistency. The predicted changes in distance distributions from MMM were all within 1.0 nm of each other, between 4.0–5.0 nm. However due to the fast decay of the signal, 4.5 nm was the upper limit of the distance distribution that could be calculated using DeerAnalysis. Therefore, the results obtained could not access long enough distances to validate MMM predictions.

Figure 4.14 shows the background corrected time decays and resultant distance distributions for Glt_{Tk} I312C in detergent micelles. Although the distances were not likely to be accurate, there was a general trend observed. Upon addition of Na⁺, the distance distribution increased which then broadened and shifted to a shorter distance upon binding substrate. The inhibitor bound distance distributions resembled those of the sodium bound state. Due to the very small distance distribution changes in MMM it was difficult to associate these with specific conformations. In MMM the outward facing state had predicted distances around 4.5–5.0 nm, with inward facing conformations at shorter distances of 4.0–4.4 nm

(Figure 4.15). The intermediate outward occluded conformation had distance distributions (4.0 - 4.1 nm) which overlapped with those of the inward facing state making it hard to distinguish between these states.

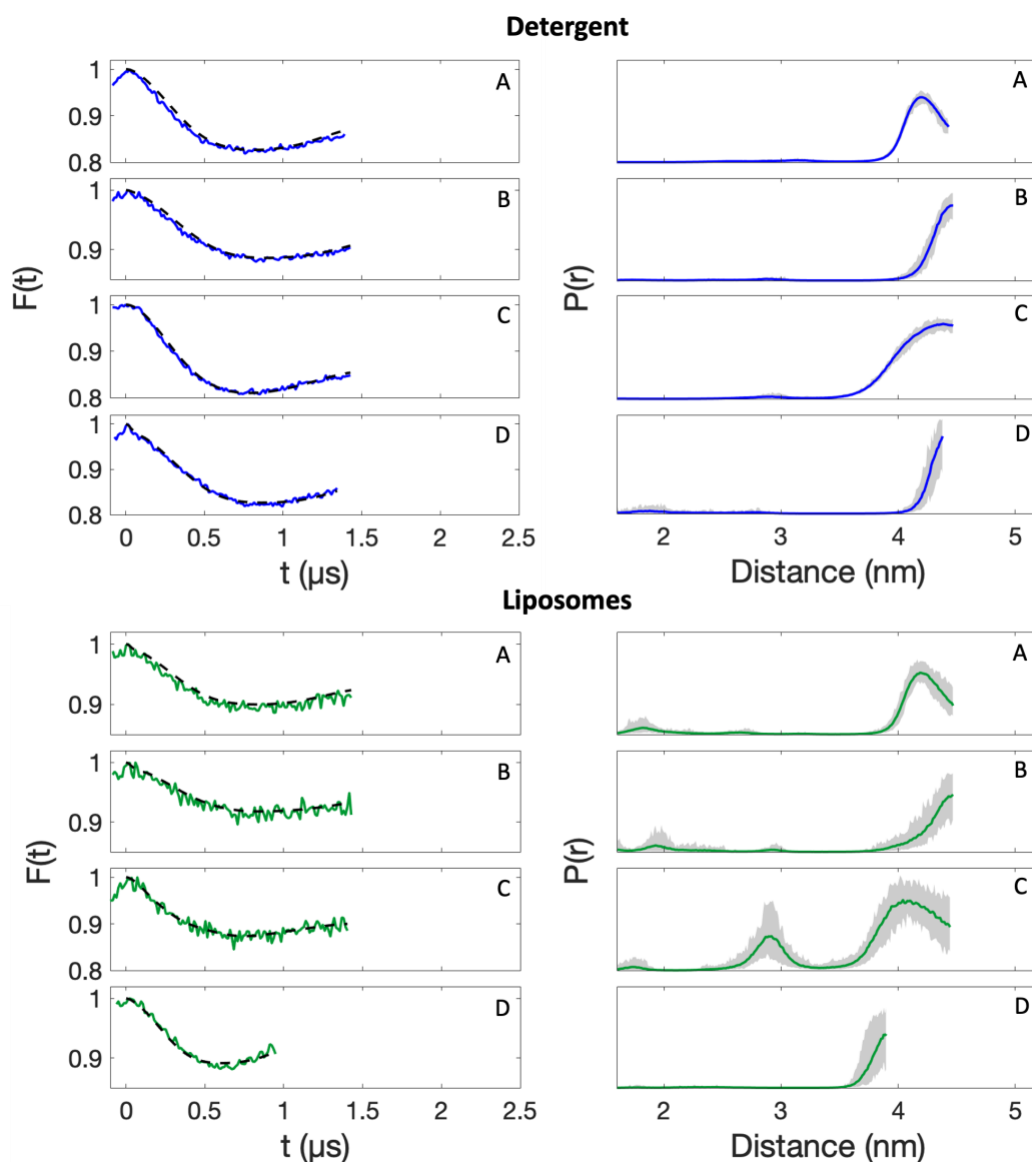


Figure 4.14 4-pulse DEER experiments of Glt_{Tk} I312C in detergent micelles (blue) and proteoliposomes (green) utilising substrate and co-transported Na⁺ to trap Glt_{Tk} in proposed intermediates of the transport cycle: A) apo, B) Na⁺ (50 mM), C) substrate bound (50 mM Na⁺ and 1 mM L-aspartate) and D) inhibitor bound (50 mM Na⁺ and 1 mM DL-TBOA). The normalised background corrected time decays are shown left with their respective fittings shown as black dashed lines. The right hand traces show the calculated distance distributions using DeerNet and the 95% confidence bounds represented by the light grey areas.

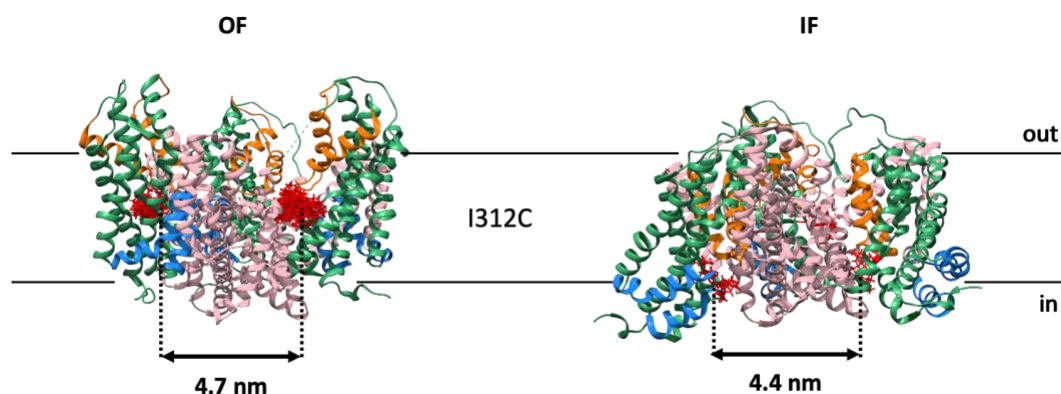


Figure 4.15 MMM results for Glt_{TK} I312C in an outward open (left, PDB 4KY0) and 2 inward open protomers with 1 intermediate outward occluded protomer (right, PDB 6XWR) with MTSL rotamers shown as red clusters. The transport domain (green) and scaffold domain (pink) are shown, with HP1 (blue) and HP2 (yellow) highlighted. The distance distribution is represented by a black arrow, and the distance shown between two protomers in the same conformation.

Although the EPR signal for Glt_{TK} I312C in proteoliposomes was weaker and the resultant fit of the background corrected time decays were poor (Figure 4.14), the same pattern was observed in terms of distance distribution changes as in detergent micelles. The only difference between the two sets of data was the presence of a second peak in the distance distribution of the substrate bound state in liposomes. However, this may not have been 'real' based on the confidence bounds and poor fit of the time decay and was almost 1.0 nm shorter than any of the inter-spin distances predicted by MMM.

The RT cw-EPR spectra had very similar spectral line shapes for apo, Na⁺ and Na⁺-substrate with the most abundant protein species in EasySpin simulations having the faster τ_c . However, in Na⁺-inhibitor bound Glt_{TK} I312C the most abundant protein species had the slower τ_c . The ratio's for all binding states indicated a mixture of conformations which was not shown in the DEER distance distributions. The exception was for substrate bound Glt_{TK} I312C in proteoliposomes where two distance peaks were present, and the broadness of distance distribution for substrate bound Glt_{TK} I312C in detergent micelles which was likely to be a variety of states. The lack of multiple distances could also be due to the limited time to record the Hahn

echo. DeerNet results showed similar distances and simulations between apo and substrate bound states, with Na⁺ and inhibitor bound also being similar.

4.5 Measuring distance changes of Glt_{TK} M314C using pulsed EPR spectroscopy

Of all the variants, Glt_{TK} M314C had the lowest yield and labelling efficiency. It is part of the NMDGT motif and is involved in binding Na⁺. The initial FSE and T₂ experiments found that Glt_{TK} M314C had the same phenomenon observed for Glt_{TK} I312C in terms of the addition of d₈ glycerol having no effect on spin-spin relaxation time of the spin (Appendix A13). Glt_{TK} M314 was proposed to be exposed in an apo state, with accessibility of Na⁺ to enter the binding site. Glycerol however is a neutral larger molecule than an Na⁺ ion which may account for the inaccessibility observed. When combined with the large distance distributions predicted by MMM (3.3–6.0 nm) this was the most challenging candidate for DEER experiments. Successfully measuring 6.0 nm distances using pulsed X-band would be challenging even for high quality samples. The distance distributions in different binding conditions were presented in Section 3.2 and are shown in Figure 4.16.

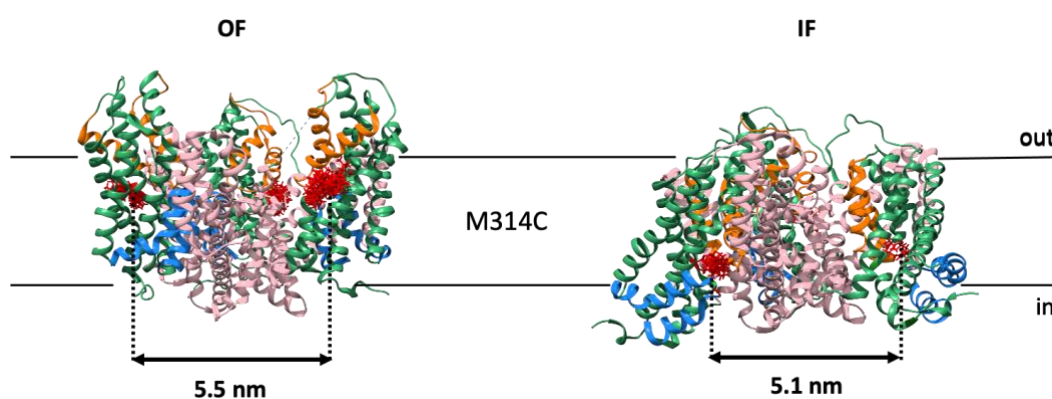


Figure 4.16 MMM results for Glt_{TK} M314C in an outward open (left, PDB 4KY0) and 2 inward open protomers with 1 intermediate outward occluded protomer (right, PDB 6XWR) with MTSL rotamers shown as red clusters. The transport domain (green) and scaffold domain (pink) are shown, with HP1 (blue) and HP2 (yellow) highlighted. The distance distribution is represented by a black arrow, and the distance shown between two protomers in the same conformation.

The short spin-spin relaxation time limited the time frame to measure the refocused Hahn echo, restricting the distance distributions to a maximum of 4.2 nm using DeerNet. This was below most of the predicted distances using MMM except for an intermediate outward occluded state when bound to L-aspartate. The substrate bound structure in an outward occluded state had predicted distances >4.2 nm, so an observable distance was unlikely depending on the conformation the protein was in. The DeerNet results are shown in Appendix A14. These results were not reliable due to the large areas of uncertainty, and introduction of oscillations in the fit (which were not present in the experimental data) as a result of low signal to noise. Glt_{Tk} M314C in proteoliposomes had even more noise, again with poorly fitted data and unreliable distance distributions. Overall, Glt_{Tk} M314C was not a good candidate for pulsed EPR studies. The presence of multiple conformations seen in cw-EPR spectra cannot be ruled out due to the poor quality of the data and limited distance range.

4.6 Measuring distance changes of Glt_{Tk} H334C using pulsed EPR spectroscopy

The RT cw-EPR spectra had subtle changes in spectral line shape between the binding states. EasySpin simulation results revealed changes in τ_c of the two protein species indicating conformational changes had happened. Pulsed EPR was used to investigate this further by looking at changes in distance distribution in both detergent micelles and proteoliposomes. The predicted distance distributions of Glt_{Tk} H334C during the transport cycle ranged between 3.6–7.5 nm (Figure 4.17 and Section 3.2). Distances of 7.5 nm are difficult to measure using DEER experiments, especially when studying membrane proteins; 3.6 nm was also the only distance <4.0 nm predicted, and it was for a single protomer in the Na⁺ bound state which has two different conformations (2 inward open and 1 intermediate outward occluded).

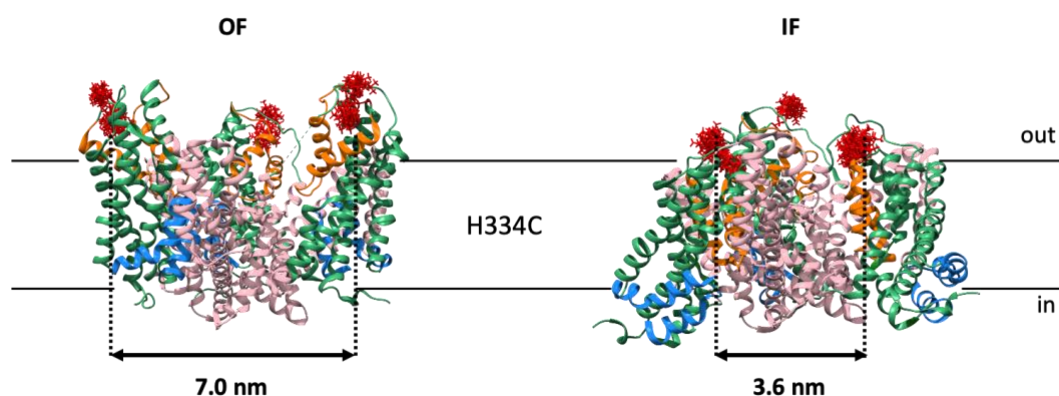


Figure 4.17 MMM results for Glt_{Tk} H334C in an outward open (left, PDB 4KY0) and 2 inward open protomers with 1 intermediate outward occluded protomer (right, PDB 6XWR) with MTSL rotamers shown as red clusters. The transport domain (green) and scaffold domain (pink) are shown, with HP1 (blue) and HP2 (yellow) highlighted. The distance distribution is represented by a black arrow, and the distance shown between two protomers in the same conformation.

The Hahn echo for Glt_{Tk} H334C was detectable for 2.5 μ s which equates to a maximum observable spin-spin separation of 5.0 nm using DeerNet. Based on MMM predictions using cryo-EM structures in different conformations, inward facing Glt_{Tk} H334C has shorter distances between spin labels $\sim$ 4 nm, and the higher end of the predicted range (7.5 nm) corresponds to outward facing conformations; with intermediate occluded states in the middle ($\sim$ 5 - 6 nm). The results for detergent micelles showed few distances in the range of distance distributions that could be measured, with partial peaks cut off at the end (Figure 4.18). The predicted distance of 3.6 nm for the Na⁺ bound state appeared as a small peak within the degree of uncertainty, and the longer distance of 5.0 nm. These corresponded to distances predicted for an inward open and intermediate occluded conformation respectively. The latter was present in all four binding states at the cut off distance. This suggested the limitation of these results was likely to be from the protein concentration and relaxation time. All other binding states had predicted distances >5.0 nm which could not be reached under the experimental set up used.

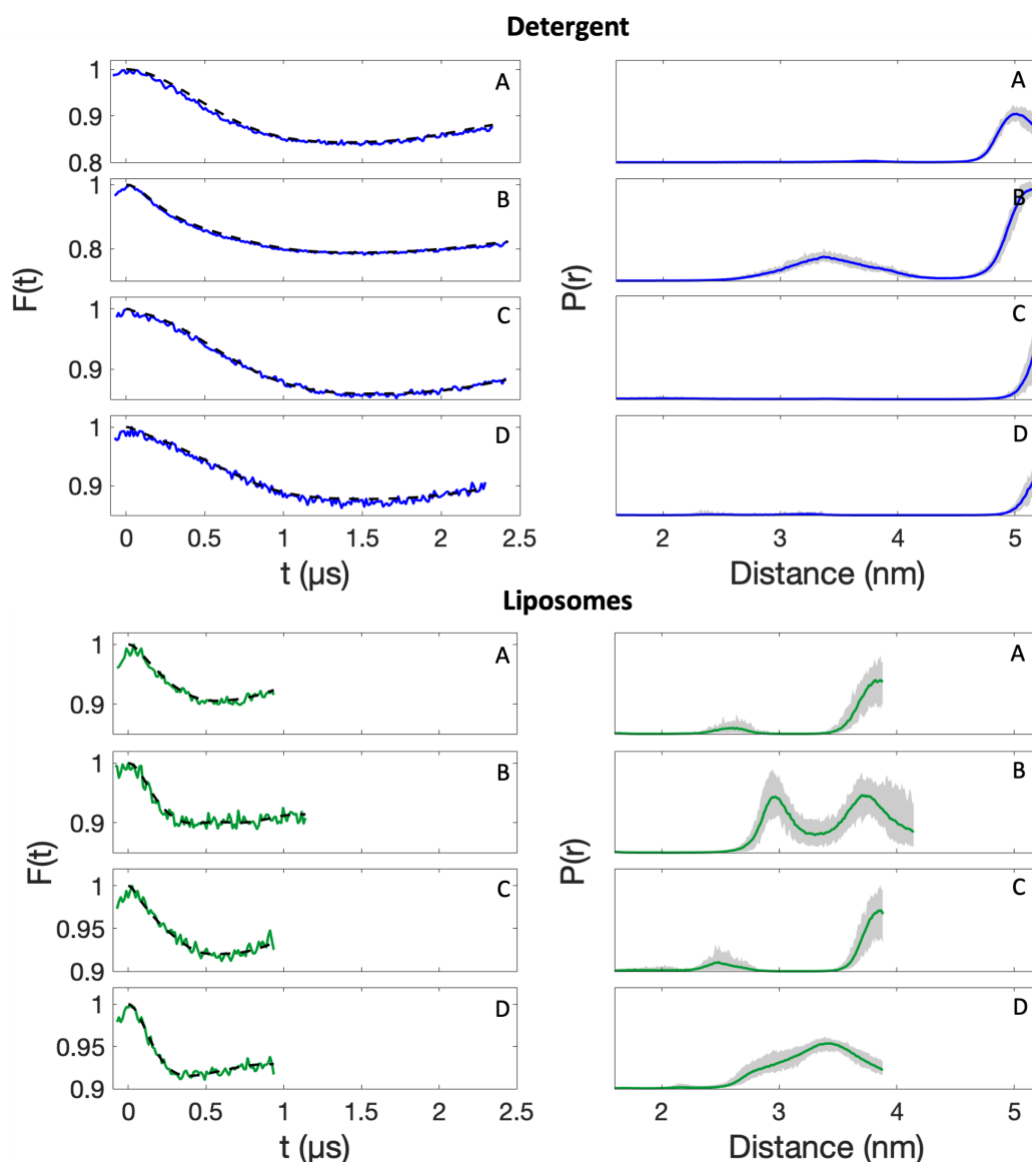


Figure 4.18 4-pulse DEER experiments of Glt_{T_k} H334C in detergent micelles (blue) and proteoliposomes (green) utilising substrate and co-transported Na⁺ to trap Glt_{T_k} in proposed intermediates of the transport cycle: A) apo, B) Na⁺ (50 mM), C) substrate bound (50 mM Na⁺ and 1 mM L-aspartate) and D) inhibitor bound (50 mM Na⁺ and 1 mM DL-TBOA). The normalised background corrected time decays are shown left with their respective fittings shown as black dashed lines. The right hand traces show the calculated distance distributions using DeerNet and the 95% confidence bounds represented by the light grey areas.

Due to the lower protein concentration and fast relaxation of Glt_{T_k} H334C proteoliposomes, only distances up to 4.0 nm could be calculated for these samples. If the noise in the trace becomes the same order of magnitude as the modulation depth, DeerNet can misinterpret noise as oscillations giving spurious distance distributions. This is proposed to have occurred in Figure 4.18 where the fits of the

background corrected time decays contained oscillations which were unlikely to be present within the noise of the signal. This was particularly evident for the Na⁺ state in both the oscillation in the fit at 0.5 and 1 μ s where there are higher levels of noise in the trace and the distances predicted which correspond to this.

4.7 Measuring distance changes of Glt_{Tk} A367C using pulsed EPR spectroscopy

Glt_{Tk} A367 is part of HP2, a gate to the intra- and extracellular environment (refer to Section 1.5.1). RT cw-EPR spectra and their simulations showed obvious changes under the different binding conditions and a bigger difference between the three environments compared to Glt_{Tk} S281C. SMALPs showed less significant changes compared to detergent micelles and proteoliposomes. Glt_{Tk} A367C was studied in the three environments (detergent micelles, proteoliposomes and SMALPs) using DEER experiments to measure the binding induced changes in the distance distributions. The predicted distance changes using MMM ranged between 2.6–5.0 nm which were reasonable to experimentally determine using pulsed EPR (Figure 4.19 and Section 3.2). The time decay traces plus fits of the data used to predict distance distributions are shown in Figure 4.20.

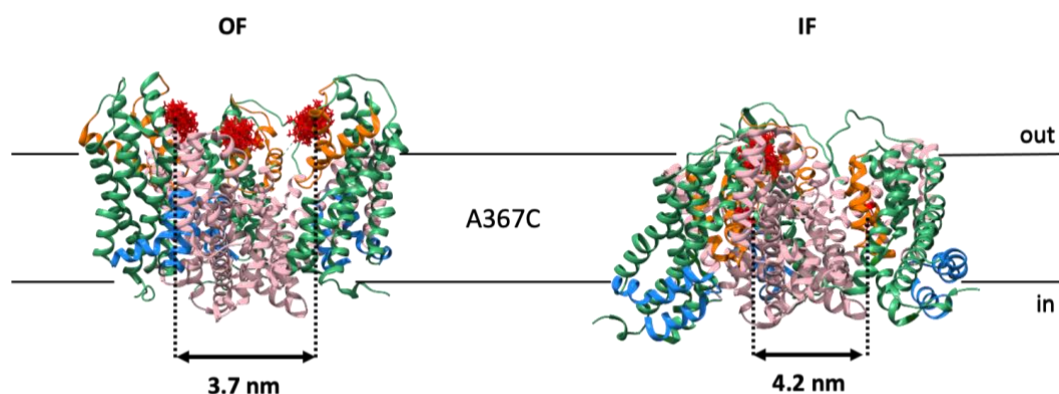


Figure 4.19 MMM results for Glt_{Tk} A356C in an outward open (left, PDB 4KY0) and 2 inward open protomers with 1 intermediate outward occluded protomer (right, PDB 6XWR) with MTSL rotamers shown as red clusters. The transport domain (green) and scaffold domain (pink) are shown, with HP1 (blue) and HP2 (yellow) highlighted. The distance distribution is represented by a black arrow, and the distance shown between two protomers in the same conformation.

The difficulty of obtaining comparable time delays in proteoliposomes (due to protein concentration) was evident again for this set of data. This was also the case for SMALPs and restricted the distances that could be determined using DeerNet for the more native environments. The results obtained in detergent micelles follow the trend of those predicted by MMM, although all distance peaks were relatively broad indicating a range of similar distances (and conformations) as opposed to a uniform single-distance peak. Apo Glt_{TK} A367C in an outward open conformation was expected to give distances of ~3.7 nm but the distances observed using DEER appeared as a broad peak between 2.5–3.5 nm. The larger end of the distances are proposed to correspond to the outward open conformation predicted but at 0.2 nm lower than expected. In an intermediate outward occluded state, the distance distribution for Glt_{TK} A367C was predicted to be ~2.6 nm which corresponded to the lower end of the range observed for apo. Upon binding sodium, a shift to 4.2–4.5 nm was predicted by MMM for an inward facing conformation. After addition of Na⁺ a peak around 4.0 nm appeared, which also appeared 0.2 nm lower than MMM predictions.

When substrate bound, a new peak at 2.5 nm appeared which represented the intermediate outward occluded state predicted by MMM at 2.6–2.7 nm, in addition to an outward open peak at 3.5 nm. Results for inhibitor bound Glt_{TK} A367C had the poorest fit of the detergent micelle data for this variant. The resulting distances were across a broad range which correspond to outward facing states. MMM predicted distances of 4.0, 4.6 and 5.0 nm so the experimental distances appeared lower than this, with the exception of the 5.0 nm peak which was cut off. The broad distances between 3.5 nm and 4 nm could represent a mixture of outward and inward open conformations, correlating with observations in the other binding states. Overall, these results were less clear than Glt_{TK} S281C, but a general trend and correlation was seen, which is why this variant was selected as the second candidate to study in SMALPs.

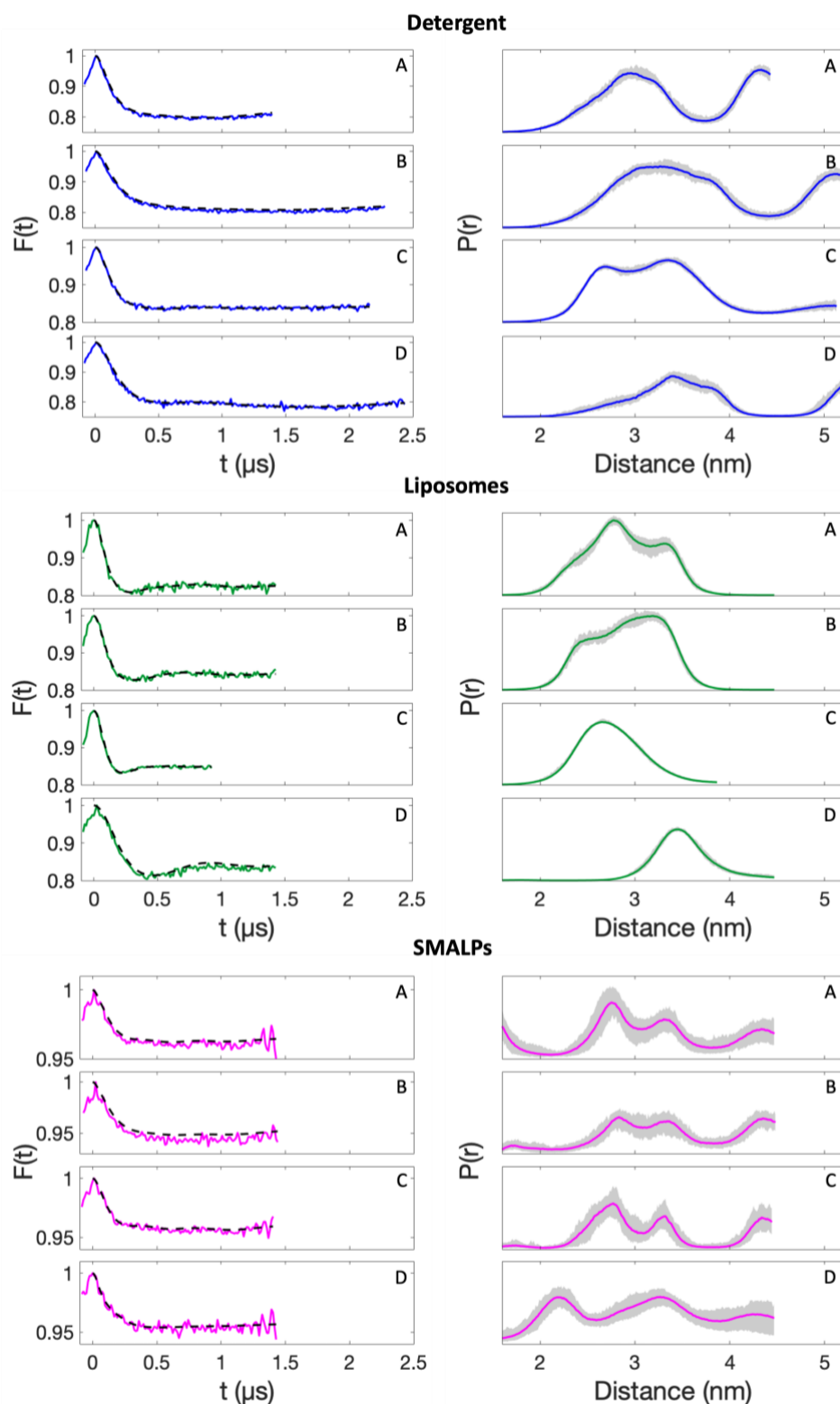


Figure 4.20 4-pulse DEER experiments of Glt_{TK} A367C in detergent micelles (blue), proteoliposomes (green) and SMALPs (pink) utilising substrate and co-transported Na⁺ to trap Glt_{TK} in proposed intermediates of the transport cycle: A) apo, B) Na⁺ (50 mM), C) substrate bound (50 mM Na⁺ and 1 mM L-aspartate) and D) inhibitor bound (50 mM Na⁺ and 1 mM DL-TBOA). The normalised background corrected time decays are shown left with their respective fittings shown as black dashed lines. The right hand traces show the calculated distance distributions using DeerNet and the 95% confidence bounds represented by the light grey areas.

Although the fits in Figure 4.20 of the time decays for Glt_{TK} A367C in proteoliposomes were good, the experimental set up meant only distances of up to 4.0 - 4.5 nm could be determined using DeerNet. All peaks in the output were <3.5 nm, with apo and Na⁺ having broad distributions like in detergent micelles. In an apo state this was likely to mean the protein was sampling multiple conformations including outward occluded and intermediate outward occluded states based on MMM predictions (which was the same as detergent micelles). When Na⁺ was added the distance distribution shifted to include a larger population of the longer distances ~3.5 nm. This was below 4.2–4.5 nm predicted by MMM for this state. Substrate bound Glt_{TK} A367C in proteoliposomes had a much narrower distribution compared to detergent and corresponds to the MMM predictions for the intermediate outward occluded state. In the presence of inhibitor this shifted to a narrower distribution at 3.5 nm which again was shorter than the predicted 4.0–5.0 nm range. This inhibitor bound state did not exhibit the asymmetric behaviour observed for Glt_{TK} S281C, but the distances representing Na⁺-inhibitor bound, inward facing Glt_{TK} A367C could have been present beyond the distances measured. The distance distributions extracted for Glt_{TK} A367C showed the correlation to intermediate outward occluded conformations at 2.7 ns, outward open around 3.4 ns and inward open around 4.1 ns. However, the distance distributions had a higher level of uncertainty due to the increased noise levels of the time traces. The distance distributions extracted for Glt_{TK} A367C in SMALPs showed correlation with proteoliposomes and detergent micelles, however the signals were weaker (lower modulation depth) and therefore the time traces had higher noise levels.

5 Utilising engineered double histidine motifs for copper (II) EPR studies of Glt_{ph}

Previous EPR studies of Glt_{ph} aimed to probe conformational changes in the presence of sodium ions and substrate, or inhibitor molecules using continuous wave (cw) and pulsed EPR techniques. Particular focus surrounded the movements and relocation of the extracellular gate, HP2, in different binding states which was not clear in early crystal structures.^{142,145} All of the published EPR studies utilised MTSL as the chosen spin probe for the cysteine variants.^{134,135,142,144,145} However, EPR studies of other proteins have found the use of copper (II) nitrilotriacetic acid (Cu²⁺-NTA) as an alternative spin label yields distance distributions 5 times narrower than the more widely employed nitroxide spin labels such as MTSL^{187,188}. The increased precision was a direct result of the chelation of Cu²⁺-NTA by a double histidine (dHis) motif which restricted the conformational flexibility (Figure 5.1). The attachment of MTSL to a single cysteine residue means results are often dominated by the motion and flexibility of the spin-label as opposed to the protein backbone,^{188,189} making the use of alternative spin labels an area of interest in studying protein conformational changes. Using an orthogonal labelling approach (i.e. two or more types of spin labels) expands the information accessible using biophysical techniques.

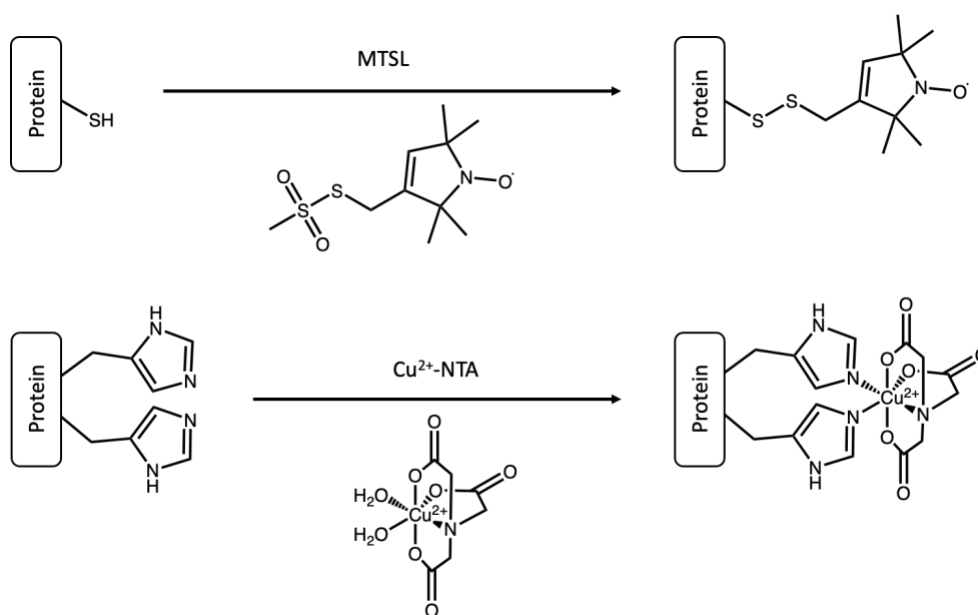


Figure 5.1 Spin-labelling protein using MTSL (top) and Cu²⁺-NTA (bottom). MTSL binds to cysteine residues via a disulfide bridge. The double histidine motif on the protein coordinates Cu²⁺-NTA as an alternative spin-label.

The requirement of a dHis motif for Cu²⁺-NTA means, unless present in the native protein, it needs to be introduced using mutagenesis. This is a particularly useful method if the protein of interest contains functionally important native cysteine residues which would make attachment of MTSL via a disulfide challenging. Some proteins, such as metalloproteins, may contain native dHis motifs but this was not the case for Glt_{ph}. The location of the dHis motif is particularly important, with an i, i+4 motif needed for alpha helices and i, i+2 motif for beta sheets to specifically and rigidly coordinate the Cu²⁺ complex (Figure 5.2).^{188,190,191}

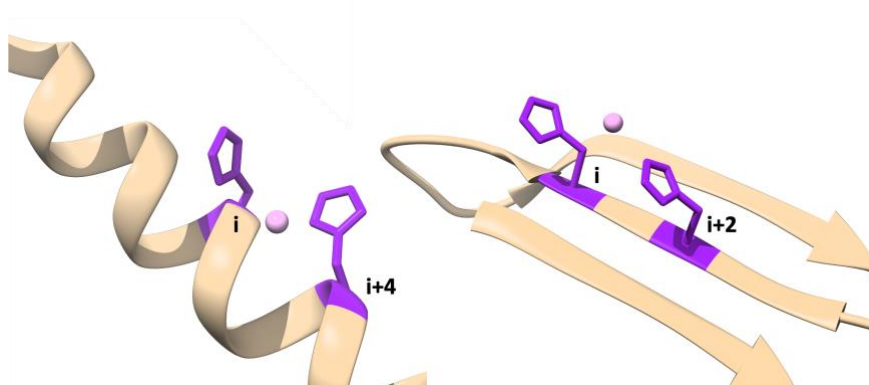


Figure 5.2 Binding of Cu²⁺ to a dHis motif on alpha helix (left) and beta sheet (right). Copper is represented by a pink sphere, and the histidine residues in purple. The dHis location differs with secondary structure: alpha helices require an i, i+4 arrangement and beta sheet requires i, i+2.

This chapter presents initial studies of Cu²⁺-NTA with Glt_{ph} as an alternative spin probe to MTSL. The ultimate aim of this project was to apply inversion-recovery-filtered DEER (iDEER) spectroscopy to simplify the distance distributions observed for homotrimeric Glt_{ph}. For these studies, an orthogonal labelling approach was utilised where Glt_{ph} was spin-labelled using both MTSL and Cu²⁺-NTA. The use of iDEER would then allow distances to be eliminated between pairs of like sites based on their relaxation times (refer to Appendix A15). Initial experiments of a Glt_{ph} variant were conducted with each spin label separately, and then together, with the future outlook of conducting iDEER studies. The study in which iDEER was first reported involved a nitrite reductase protein which had two native copper binding sites,¹⁹² as opposed to the two non-native sites used for this project.

5.1 Cu²⁺ EPR theory

Cu²⁺ EPR spectroscopy differs to that of nitroxide radicals. Copper typically forms Cu⁺ and Cu²⁺ ions which have 10 and 9 d-electrons respectively. The 3d shell is fully occupied and Cu⁺ is therefore EPR silent. The more common Cu²⁺ has an unpaired electron giving rise to $S = 1/2$ and is therefore EPR active. The naturally occurring isotopes of Cu (⁶³Cu and ⁶⁵Cu) have a nuclear spin of $3/2$ giving rise to a nuclear Zeeman interaction.^{151,193} The hyperfine coupling results in splitting of the EPR signal into four lines, compared to the three lines seen for MTSL. However, the EPR spectrum varies depending on the coordination geometry of the Cu²⁺ ion. For Cu²⁺ there are two principal g-values typically observed: $g_{||}$ (g_{zz}) and $g_{\perp}$ (g_{xx} and g_{yy}).¹⁹³ The sensitivity of EPR for the coordination geometry is demonstrated by Gamble et al. who fitted an experimental cw-EPR spectrum of Cu²⁺-NTA using two different coordination geometries to successfully replicate the line shape (Figure 5.3).¹⁹¹ In addition to $g_{||}$ and $g_{\perp}$, the EPR spectrum contains the hyperfine splittings $A_{||}$ and $A_{\perp}$. Due to large line widths and small $A_{\perp}$, the $g_{\perp}$ region may not be resolved, but $g_{||}$ and $A_{||}$ can be determined. Therefore EPR spectra can be used to determine the coordination environment of the metal i.e. four, five or six coordinate by their g-values and A-values.¹⁵¹

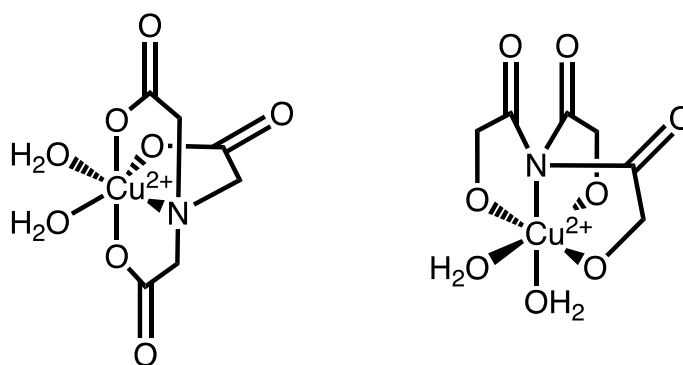


Figure 5.3 The two coordination modes of Cu²⁺-NTA found in previous EPR studies, where the left hand structure was the dominant species.

Free Cu²⁺ has poor selectivity and forms complexes with many ligands, increasing the risk of non-specific coordination if still present. The presence of free Cu²⁺ also results in the degradation of signal in PELDOR experiments, highlighting the importance of

carefully selecting the ratio of Cu²⁺-NTA:protein.¹⁹¹ To measure the distance distribution between pairs of spin probes, PELDOR is commonly used, particularly for nitroxide labelled protein samples. For Cu²⁺ labelled samples a smaller part of the spectrum is excited, compared to MTSL, due to the limited bandwidth of the microwave pulses. This means PELDOR experiments often exhibit shallower modulation depths and limited sensitivity. Relaxation-induced dipolar modulation enhancement (RIDME) is an alternative pulsed EPR technique more commonly used for Cu²⁺ labelled species due to the increased sensitivity. A single frequency is used with a spontaneous spin flip to refocus the Hahn echo, as opposed to a microwave pulse in PELDOR.^{194,195}

5.2 *In silico* molecular modelling of Glt_{ph}

A selection of Glt_{ph} variants were available from collaborators which contained both cysteine and dHis sites. These variants were originally created to study distance distributions using transition metal FRET between a fluorescein labelled cysteine and copper or nickel coordinated dHis motif. Their suitability for EPR studies was assessed using *in silico* modelling (MMM) for spin labelling with both MTSL and Cu²⁺-NTA. The results for Glt_{ph} CM7 (C321S G149C V51H K55H) are shown in Figure 5.4 and Table 5.1 with the other variants included in Appendix A16. *In silico* analysis using Cu²⁺-NTA required a modified method (see Appendix A17) whereby residues were bilabelled with NTA to ensure attachment to both histidine residues (dHis at i, i+4) by one label.¹⁸⁸

Distances between 2–8 nm can be measured using DEER experiments, as demonstrated previously for Glt_{TK}. When distances <2 nm are present, it can be visible in low temperature (LT) cw-EPR spectra as dipolar broadening. These shorter distances could then be extracted using a software called pyDipfit if a singly labelled sample is produced.¹⁹⁶ However for Glt_{TK} and Glt_{ph} this is not applicable as the sample conformations are not homogeneous for the trimeric proteins. The lowest predicted distance for Glt_{ph} CM7 was 1.60 nm which is at the upper limit of detection so it is

unlikely the dipolar broadening would be visible anyway. For a trimeric structure it is impossible to determine a singly labelled line shape which is needed for the input of pyDipfit.

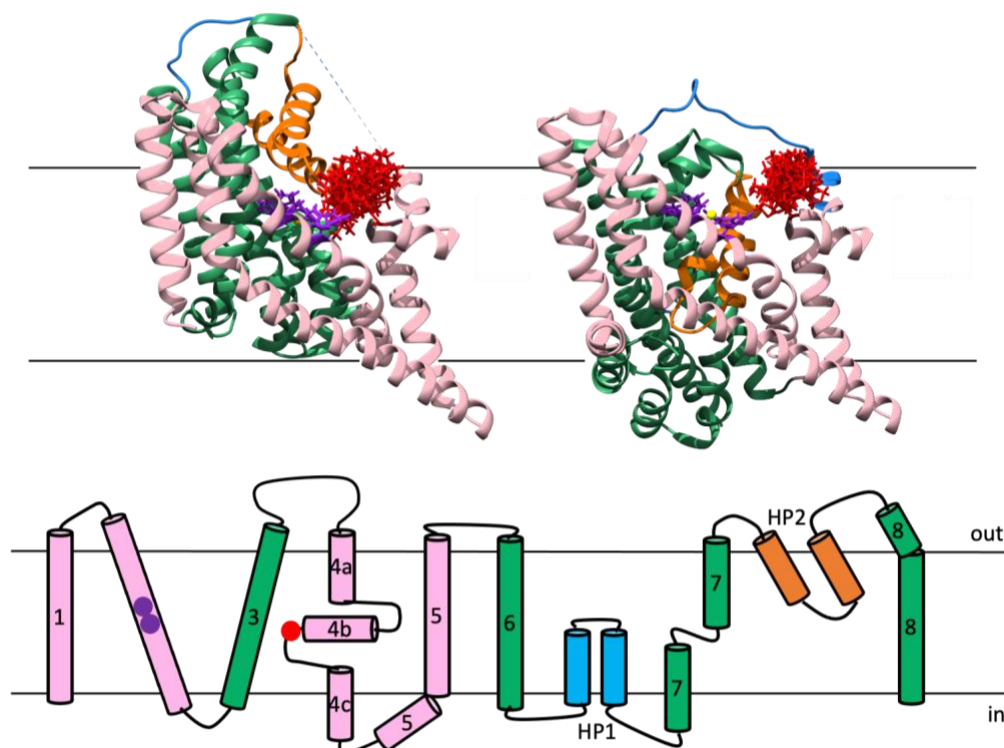


Figure 5.4 Glt_{ph} membrane topology and structure. It is composed of a transport domain (green) and scaffold domain (pink), with HP1 (blue) and HP2 (orange) highlighted. Top: A Glt_{ph} protomer in an outward open state (left, PDB 40YF) and inward facing (right, PDB 6X15). Red clusters represent attachment of MTSL, and purple clusters are attachment of Cu²⁺-NTA to Glt_{ph} CM7 using *in silico* modelling. Bottom: Glt_{ph} CM7 membrane topology with the MTSL attachment site as a red dot, and the dHis motif as two purple dots.

Glt_{ph} CM7 contains both points of spin-label attachment (the cysteine residue and dHis motif) within the scaffold domain. During the alternating access mechanism, the transport domain moves relative to the rigid scaffold domain. G149 is located on an unwound region between TM4b and TM4c which may have limited flexibility. This contrasts to the dHis motif located in the middle of TM2 which would be expected to remain fixed during the transport cycle. The other variants are shown with their topology diagrams in Appendix A16, where the cysteine residue for attachment of MTSL, and dHis motif for Cu²⁺-NTA coordination are located on different regions of the protein.

State	Conformation(s)	Spin Label	Rotamers	Distances (nm)
Apo ^I	Outward facing occluded	MTSL	128 / 127 / 129	2.27 / 2.27 / 2.26
		Cu ²⁺ -NTA	11 / 12 / 11	3.25 / 3.26 / 3.26
		MTSL & Cu ²⁺ -NTA	-	3.08 / 2.37 / 2.36 / 3.08 / 3.08 / 2.35
Apo ^{II}	Inward facing occluded	MTSL	53 / 55 / 58	1.65 / 1.60 / 1.67
		Cu ²⁺ -NTA	6 / 8 / 9	3.38 / 3.37 / 3.40
		MTSL & Cu ²⁺ -NTA	-	2.72 / 2.48 / 2.42 / 2.82 / 2.67 / 2.41
Na ⁺ only ^{III}	Outward occluded	MTSL	120 / 125 / 125	2.48 / 2.50 / 2.47
		Cu ²⁺ -NTA	16 / 16 / 14	3.23 / 3.22 / 3.22
		MTSL & Cu ²⁺ -NTA	-	3.20 / 2.40 / 2.38 / 3.17 / 3.19 / 2.39
Substrate bound ^{IV}	Inward facing occluded	MTSL	68 / 69 / 68	2.15 / 2.18 / 2.14
		Cu ²⁺ -NTA	18 / 17 / 18	3.46 / 3.46 / 3.45
		MTSL & Cu ²⁺ -NTA	-	3.01 / 2.68 / 2.68 / 3.01 / 3.02 / 2.66
Substrate bound ^V	Inward facing	MTSL	50 / 49 / 49	2.47 / 2.46 / 2.47
		Cu ²⁺ -NTA	5 / 5 / 5	3.42 / 3.42 / 3.42
		MTSL & Cu ²⁺ -NTA	-	3.20 / 2.62 / 2.62 / 3.19 / 3.20 / 2.62
Inhibitor bound ^{VI}	Inward facing	MTSL	82 / 83 / 81	1.66 / 1.68 / 1.77
		Cu ²⁺ -NTA	6 / 6 / 6	3.36 / 3.37 / 3.39
		MTSL & Cu ²⁺ -NTA	-	2.93 / 2.19 / 2.04 / 2.91 / 2.88 / 2.08
Inhibitor bound ^{VII}	Outward facing	MTSL	104 / 99 / 92	2.30 / 2.59 / 2.48
		Cu ²⁺ -NTA	7 / 8 / 9	3.34 / 3.31 / 3.30
		MTSL & Cu ²⁺ -NTA	-	3.22 / 2.43 / 2.32 / 3.08 / 3.35 / 2.49

Table 5.1 Results for MMM analysis of Glt_{ph} CM7 (C321S G149C V51H K55H) using structural models. Each model was analysed using both MTSL and Cu²⁺-NTA as spin-labels for cysteine and dHis motif respectively. Predictions of the number of rotamers on each protomer (A/B/C) and the distance distributions between pairs of residues (AB, AC, BC) are displayed. Results given for bilabelled Glt_{ph} CM7 are inter-protomer distances between heterogeneous pairs of labels, so these are in addition to the distances obtained for each type of spin-label separately. All intra-protomer distances were <2nm for this variant. A range of binding states and conformations were studied to see how the selected position moves during the transport cycle where substrate bound refers to sodium and L-aspartate, and inhibitor is with sodium and DL-TBOA. ^IPDB 4OYE, ^{II}PDB 4P19, ^{III}PDB 4OYF, ^{IV}PDB 3KBC, ^VPDB 6X15, ^{VI}PDB 6X16, ^{VII}PDB 2NWW

MTSL-labelled Glt_{ph} CM7 had distances ranging from 1.60–2.69 nm, with the shorter distances corresponding to inward facing conformations. However, when in an inward occluded conformation, the distance distribution was >2 nm which is within the range for pulsed EPR distance measurements. Each binding state, except Na⁺ bound, had published structures with Glt_{ph} in both inward and outward facing conformations, which has also been shown in previous EPR studies.^{134,135} Therefore, a mixture of distances were expected to be observed experimentally, but these would be subtle and within a 1 nm range. For all conformations of Glt_{ph} CM7 there was a large number of MTSL rotamers indicating that G149C was accessible for successful attachment of MTSL. The significant reduction in the number of rotamers for Cu²⁺-NTA labelled Glt_{ph} CM7 was evident - at least 10 times less than MTSL. The coordination of Cu²⁺-NTA by two histidine residues, as opposed to a single cysteine residue for MTSL, resulted in a much more rigid label hence the smaller number of calculated rotamers. Like MTSL, there were very subtle changes in distance distribution (~0.2 nm) in the different conformations which would be challenging to observe experimentally. The relatively fixed distance distribution is likely to be a result of the location of the dHis motif on TM2 in the scaffold domain.

The most interesting results when comparing the Glt_{ph} variants was when labelling with both MTSL and Cu²⁺-NTA. The predicted number of rotamers for each label at each site remained the same, but the presence of two spin-labels per protomer resulted in significantly more distance distributions. In addition to the six inter-protomer distances between like spin-labels, there were also six inter-protomer distances between heterogeneous spin-labels shown in Table 5.1 and three intra-protomer distances presented in Appendix A16. All Glt_{ph} variants, except Glt_{ph} CM7, had intra-protomer distances which would be measurable using pulsed EPR spectroscopy i.e. >2 nm. Therefore, all initial experiments for this research were conducted on Glt_{ph} CM7 which was predicted to have fewer distances >2 nm and therefore was predicted to produce the least convoluted distributions.

Of the other variants presented in Appendix A16, the only other interesting result was the difficulty obtained when binding Cu²⁺-NTA to Glt_{ph} CM3 (C321S G280C F50H

L54H). Glt_{ph} does not contain a native dHis residue or bind Cu²⁺ as a substrate. Therefore, the dHis motif and Cu²⁺ spin label was introduced computationally in MMM, like the mutagenesis to cysteine residues and subsequent attachment of MTSL for Glt_{Tk}. Several Glt_{ph} cryo-EM and x-ray crystallography structures were analysed in different binding conditions. Of the 8 structures analysed using MMM, only the substrate bound structure of Glt_{ph} CM3 in an inward-facing state could successfully bind the spin label. This may be a result of the relative orientations and position of the two histidine residues on the alpha helix; particularly when considering the dHis motif of Glt_{ph} CM7 and Glt_{ph} CM8 (C321S T375C V51H K55H) was one residue along. Glt_{ph} CM10 (C321S G351C K132H D136H) and Glt_{ph} CM11 (C321S R52C A364H E368H) also failed to attach Cu²⁺-NTA in one of the crystal structures, further supporting the use of Glt_{ph} CM7 as the main candidate for the initial experiments.

5.3 Glt_{ph} sample preparation

All protein production and purification was conducted using TALON Metal Affinity Resin. For MTSL-labelled samples, incubation with spin-label occurred whilst the protein was bound to the resin. This was found to be optimal in removing the maximum amount of unbound MTSL for Glt_{Tk} whilst reducing the number of steps. The protein yield for all Glt_{ph} variants was lower than those of Glt_{Tk} and varied between 0.15–0.25 mg/L, where the majority of Glt_{Tk} yields were towards the upper end of this range (Table 5.2).

The spin-labelling efficiency of each Glt_{ph} variant was calculated using the same methodology described in Section 3.3 for Glt_{Tk}. A series of TEMPO standards were recorded, and the double integration values were used to create a calibration curve. The concentration of MTSL in each sample was estimated based on the double integral of the EPR signal compared to those of TEMPO standards. This was divided by the protein concentration to calculate the labelling efficiency (Table 5.2). The efficiency of Glt_{ph} CM3 (100%) may be a result of the presence of free-MTSL, but all variants generally had good labelling efficiencies. MMM results identified Glt_{ph} CM7

as being the best candidate to study in terms of predicted distance distributions, and these results support this with a good yield and labelling efficiency.

	CM3	CM7	CM8	CM10	CM11
Yield (mg/L)	0.15	0.19	0.25	0.22	0.15
Labelling efficiency (%)	100	81	85	72	80

Table 5.2 Glt_{ph} protein yield for each variant and the calculated spin labelling efficiency ($\epsilon = 26820 \text{ M}^{-1} \text{ cm}^{-1}$, $M_w = 44608.25 \text{ g/mol}$)

5.4 EPR studies of MTSL labelled Glt_{ph}

5.4.1 Continuous wave EPR spectroscopy of Glt_{ph} labelled with MTSL

In order to investigate the conformational changes and associated dynamics during the transport mechanism, room temperature (RT) cw-EPR studies were conducted for each of the Glt_{ph} variants. Figure 5.5 shows the spectra obtained utilising substrate (L-aspartate), inhibitor (DL-TBOA) and co-transported Na⁺ ions to trap Glt_{TK} in proposed intermediates of the transport cycle. The other Glt_{ph} variants are shown in Appendix A18.

Changes in spectral line shapes indicated changes in the local environment of the spin label, which may be a result of conformational changes. This was observed for all Glt_{ph} variants to differing extents, with all four binding states of Glt_{ph} CM11 producing almost identical cw-EPR spectra. Glt_{ph} CM7 exhibited some of the largest changes in spectral line shape indicating the spin-labelled residue experienced the greatest difference in local environment. Changes in spectral line shape on the addition of L-aspartate or DL-TBOA with co-transported Na⁺ were observed as highlighted in Figure 5.5. These could be quantified using EasySpin, as discussed and presented for Glt_{TK}.

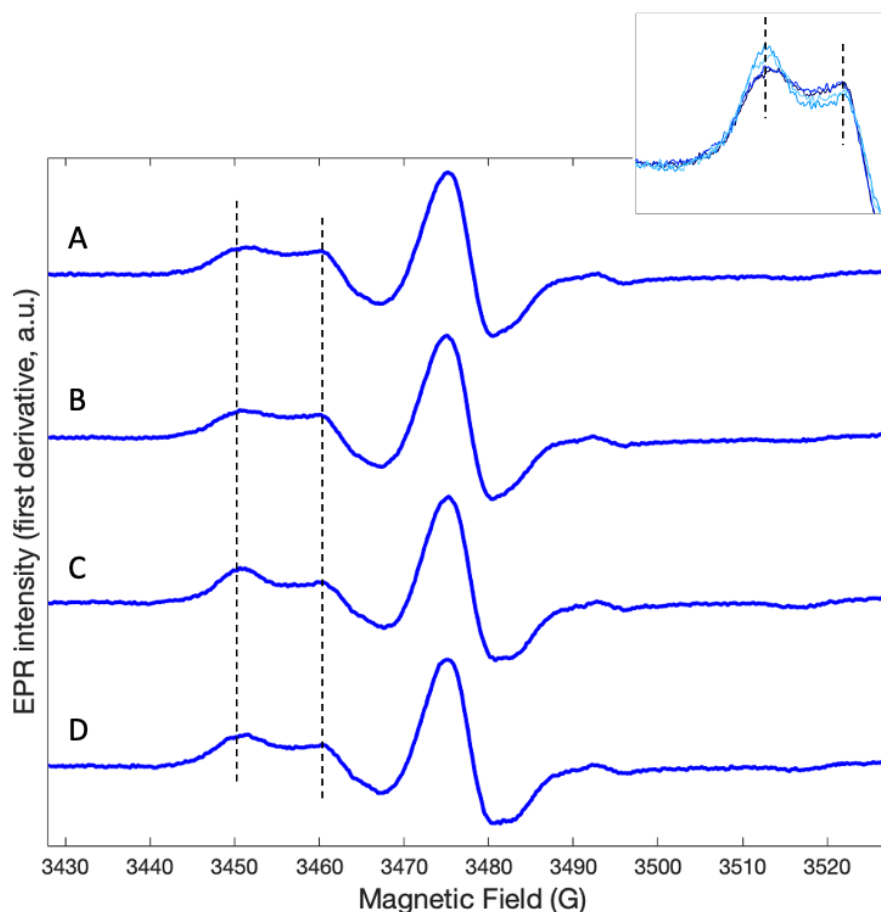


Figure 5.5 X-band RT cw-EPR spectra of Glt_{ph} CM7 in detergent micelles utilising substrate and co-transported Na⁺ to trap Glt_{ph} in proposed intermediates of the transport cycle. A) apo, B) Na⁺ (50 mM), C) substrate bound (50 mM Na⁺ and 1 mM L-aspartate) and D) inhibitor bound (50 mM Na⁺ and 1 mM DL-TBOA). Black dashed lines indicate regions of the spectra where subtle changes are observed. Inset shows an overlay of the spectrum in the region of the greatest change of line shape in response to the binding events listed above (darkest to lightest for A to D). Experimental parameters: microwave frequency = 9.758 GHz, field modulation frequency = 100 kHz, modulation amplitude = 2G, microwave power = 0.1997 mW, number averages = 100, time constant = 20.48 ms, conversion time = 20.48 ms.

All cw-EPR spectra of Glt_{ph} samples contained no obvious free-MTSL peaks, contrasting significantly to Glt_{tk} where it was present in almost every detergent micelle spectrum. Overall, these results confirmed successful attachment of MTSL to the cysteine residue on each variant, as predicted by MMM based on the high number of rotamers in different conformational states. Changes in spectral line shape indicated the protein was active and undergoing binding induced conformational changes.

5.4.2 Pulsed EPR spectroscopy of MTSL labelled Glt_{ph} for distance determination

To measure distance distributions between pairs of spin-labels, the 4-pulse DEER method was used. Initial analysis was conducted on MTSL-labelled Glt_{ph} samples at 50 K, using the same FSE and T₂ experimental set-ups as MTSL labelled Glt_{tk}. The field swept echo (FSE) was used to check the optimal observe position (i.e. the point of highest intensity, with the pump position 70 MHz downfield; displayed in Figure 5.16). Determination of T₂ showed the maximum time the Hahn echo could be observed. Glt_{ph} variants were studied in the four different binding states (apo, Na⁺, Na⁺ with L-aspartate, and Na⁺ with DL-TBOA) to elucidate conformational changes during the transport cycle. Results were analysed using DeerAnalysis to extract distance distributions from the raw time traces and compared to distance distributions predicted using MMM. The normalised background-corrected time decay traces for Glt_{ph} CM7 in the different binding states are shown alongside the extracted distance distributions in Figure 5.6. The results for all other Glt_{ph} variants are presented in Appendix A19.

The predicted distance distributions for Glt_{ph} CM7 ranged between 1.6–2.6 nm in different binding states and resulting conformations. The shorter distances would not be visible using DEER spectroscopy. Interestingly in the crystal structures used for MMM analysis specific conformations presented a different distance distribution depending on the binding condition, for example apo protein in an inward occluded apo state predicted distances around 1.6 nm, however inward occluded substrate-bound protein had predicted distances at the longer end of the range for this variant and were closer to those of an outward occluded apo state. Therefore, correlating specific distance distributions obtained experimentally with conformational states was difficult. There was no significant differences in the number of rotamers to indicate the presence of substrate or inhibitor affected distance distributions based on steric interactions.

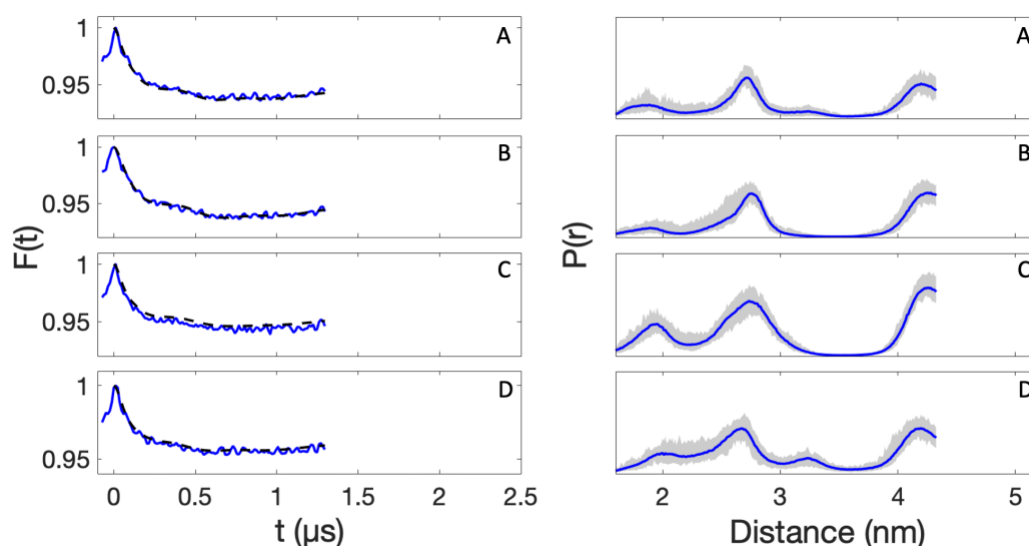


Figure 5.6 4-pulse DEER experiments of MTSL-labelled Glt_{ph} CM7 in detergent micelles utilising substrate and co-transported Na⁺ to trap Glt_{ph} in proposed intermediates of the transport cycle. A) apo, B) 50 mM Na⁺, C) substrate bound (50 mM Na⁺ & 1 mM L-aspartate), and D) inhibitor bound (50 mM Na⁺ & 1 mM DL-TBOA). The normalised background corrected time decays are shown left with their respective fittings shown as black dashed lines. The right hand traces show the calculated distance distributions using DeerNet and the 95% confidence bounds represented by the light grey areas.

The results of the T₂ experiments for all Glt_{ph} variants had a fast decay, even in the presence of d₈-glycerol. This limited the time the Hahn echo could be observed for, however with the short distance distributions predicted for this variant a long time was not required. All distance distributions obtained using DeerNet for each binding state contained a distance distribution around 4.1 nm which is significantly higher than those predicted using MMM (Figure 5.6). This is most likely an artefact of DeerNet due to the short time the Hahn echo was observed for and will not be discussed. If the quality and relaxation properties of the sample had allowed, recording over a longer observation time would clarify this.

Apo and Na⁺ bound Glt_{ph} CM7 had similar distance distributions with a peak at 2.7 nm, and a small peak around 1.8 nm which is below the reliable limit for DEER studies. MMM results (Table 5.1) predicted distance distributions of 2.3 nm for an outward occluded conformation and 1.7 nm for inward occluded apo Glt_{ph} CM7 suggesting outward-facing conformations have longer average inter-label distances.

Therefore, the observed distance at 2.7 nm is suggested to be outward open, and the shorter distance of 1.8 nm corresponding to an inward occluded state. The only crystal structure analysed for Na⁺ bound Glt_{ph} had an outward occluded conformation with similar distances to apo protein. The lack of change in distances observed experimentally in apo and Na⁺ bound suggests the same two protein conformations were present. When binding L-aspartate, a new distance appeared at 2.6 nm on the shoulder of the 2.7 nm peak seen in apo and Na⁺. There was also an increase in population at 2.0 nm which suggests larger scale conformation changes occurred when substrate bound. The presence of multiple distances may correspond to multiple protein conformations. MMM results for substrate-bound structures in an inward occluded state and outward occluded state gave similar predicted distances of 2.15–2.19 nm making it difficult to distinguish between these states. The inward facing structure predicted a longer distance of 2.47 nm, but no structure was available for outward facing substrate bound Glt_{ph} to determine how similar the inter-spin distances are (like the occluded conformations).

Inhibitor bound Glt_{ph} CM7 also showed a mixture of distances at 2.6 nm (where the peak appeared less populated than for apo and Na⁺) and smaller peaks at 2.0 nm and 3.2 nm. The longest distance predicted by MMM was 2.6 nm, so the observed distance at 3.2 nm for inhibitor bound Glt_{ph} CM7 is much larger (Figure 5.6). This could be an artefact of the higher noise levels in the time decay trace around 0.7 μs, a similar position to the predicted distance. The distance observed at 2.6 nm corresponds to the predicted distance for an outward facing conformation. This may account for the same distance seen in the substrate bound sample. Based on previous EPR studies the mixture of conformations present for each binding state was expected and suggests the protein has undergone binding induced conformational changes.^{134,135}

5.5 Spin labelling Glt_{Ph} using Cu²⁺-NTA

5.5.1 His-cleavage

Poly-histidine tags are commonly added to the N-terminus before methionine, or to the C-terminus before the stop codon, with the latter being the case for Glt_{Ph} in this project. The main purpose is for immobilized metal affinity chromatography where the poly-histidine tag binds to immobilized metal ions (including copper). The protein is eluted upon the addition of a high concentration of imidazole which competes with the his-tagged protein on the resin and displaces it. However, Cu²⁺-NTA specifically coordinates to histidine residues, so removal of the tag is required to ensure only dHis binds Cu²⁺-NTA .

The most common method for removal of poly-histidine tags is by enzymatic cleavage. Thrombin, from bovine plasma, recognises the cleavage site with the sequence L-V-P-R-G-S, cutting between the arginine (R) and glycine (G) residues just before the poly-histidine tag. The two main types of thrombin available were as lyophilized powder or as thrombin Sepharose beads. Both methods of cleavage were attempted, with the lyophilized powder proving more successful for the complete cleavage of the Glt_{Ph} poly-histidine tag. The reaction was conducted at RT overnight, with SDS-PAGE used to confirm the reaction was successful (Figure 5.7). The thrombin was inactivated by the addition of 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF) followed by the use of Zeba™ Spin desalting columns. To remove the cleaved poly-histidine tags, or any un-cleaved protein, the solution was passed over TALON Metal Affinity Resin.

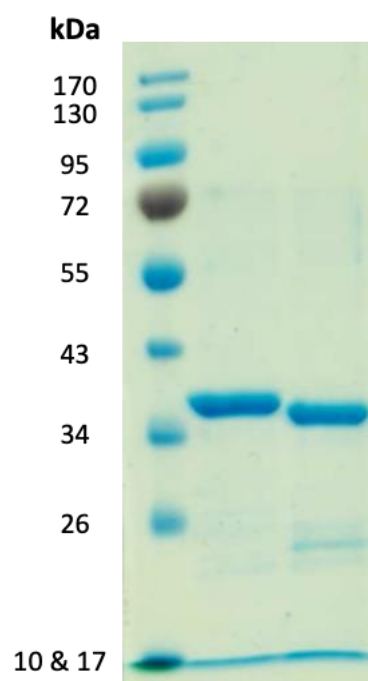


Figure 5.7 SDS PAGE of GltPh CM7 to check the poly-histidine tag cleavage using thrombin.

To ensure the removal of the poly-histidine tag didn't affect the environment of the spin-label, cw-EPR spectra were recorded for all Glt_{ph} variants in an apo state (Appendix A20). The spectral line shape for each variant remained the same, with the only difference being the presence of free-MTSL, confirming the cleavage reaction did not cause any spectral changes in the protein spectrum.

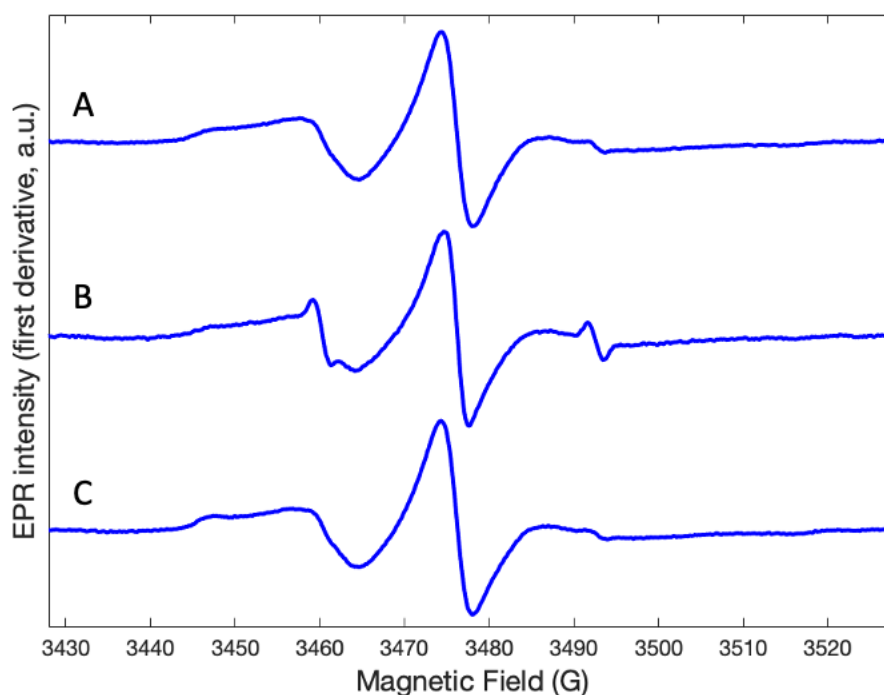


Figure 5.8 X-band RT cw-EPR spectra of apo Glt_{ph} CM3. A) spin labelled with MTSL with the poly-histidine tag attached; B) with the poly-histidine tag cleaved and subsequently spin labelled with MTSL; C) poly-histidine tag cleavage of MTSL-labelled Glt_{ph} CM3. Experimental parameters: microwave frequency = 9.758 GHz, field modulation frequency = 100 kHz, modulation amplitude = 2G, microwave power = 0.1997 mW, number averages = 100, time constant = 20.48 ms, conversion time = 20.48 ms.

The effect of the cleavage reaction on MTSL was uncertain, so a control study on Glt_{ph} CM3 was conducted where the protein was spin-labelled with MTSL either before or after the poly-histidine tag cleavage (Figure 5.8). The results confirmed MTSL remained attached during the cleavage reaction, yielding the same spectral line shape as un-cleaved protein. The spectra showed less free-MTSL was present when labelling on the column before the cleavage reaction compared to labelling the cleaved protein due to the extra wash steps in the reaction. Therefore, all

experiments requiring cleaved MTSL-labelled protein were labelled on the column as with Glt_{TK}.

As an additional control experiment to the RT cw-EPR spectra, a comparison between histidine-tag cleaved and un-cleaved MTSL-labelled Glt_{ph} was measured using low temperature (LT) X-band cw-EPR spectroscopy at 50 K. The cw-EPR spectra confirmed the local environment of the spin label remained unchanged based on the same spectral line shape between Glt_{ph} CM7 with and without the poly-histidine tag attached (Figure 5.9). This is shown for the other Glt_{ph} variants in Appendix A20.

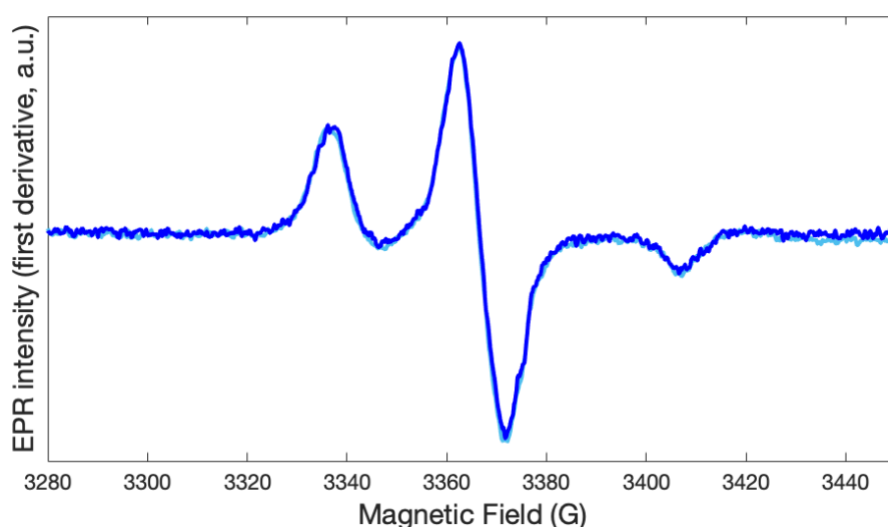


Figure 5.9 X-band LT cw-EPR spectra of Glt_{ph} CM7 (at 50 K) with and without the poly-histidine tag attached in blue and light blue respectively. Experimental parameters: microwave frequency = 9.438 GHz, field modulation frequency = 100 kHz, modulation amplitude = 2G, microwave power = 0.0125 mW, number averages = 25, time constant = 40.96 ms, conversion time = 40.96 ms.

To show no advantageous metal binding in the MTSL-labelled sample prior to Cu labelling, a LT cw-EPR spectrum was recorded with a wide field range to detect any metal signals (Figure 5.10). Functional studies of Glt_{ph} were not conducted using LT cw-EPR, as the focus of this project was on the method development of labelling Glt_{ph} with Cu²⁺-NTA, in addition to orthogonal labelling with MTSL. As with Glt_{TK}, functional studies and EasySpin simulations would confirm whether the change in line shape observed in the RT cw-EPR spectra is a result of rotational correlation time.

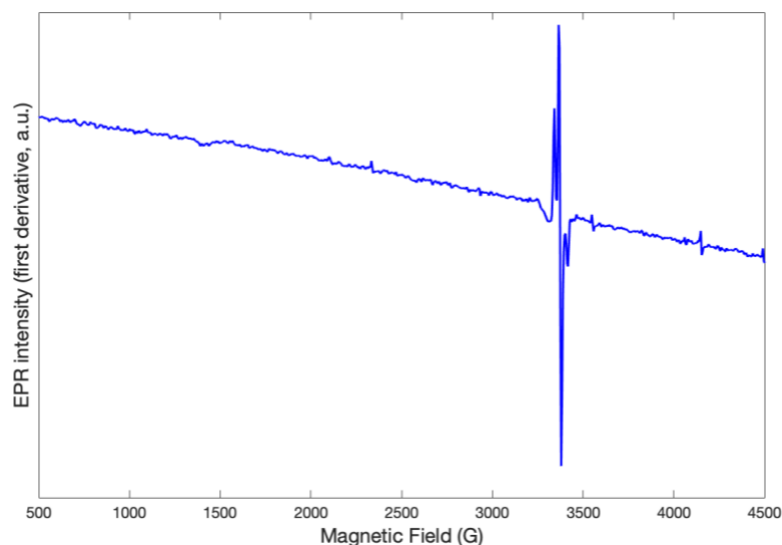


Figure 5.10 Low-temperature cw-EPR spectrum of his-cleaved, MTSL-labelled Glt_{ph} CM3 to show no advantageous metal binding. Experimental parameters: microwave frequency = 9.441 GHz, field modulation frequency = 100 kHz, modulation amplitude = 2G, microwave power = 0.9985 mW, number averages = 1, time constant = 40.96 ms, conversion time = 40.96 ms.

5.5.2 Using UV-Visible spectroscopy to check the formation of Cu²⁺-NTA

Cu²⁺-NTA was prepared from copper(II) chloride (CuCl₂) following published protocols.^{194,197} UV/Visible (UV/Vis) spectroscopy was used to monitor the formation of the Cu²⁺-NTA complex at 300 nm and 800 nm as per previous studies.^{191,198} The resulting spectra are shown in Figure 5.11 which successfully replicated published data.¹⁹¹ Previous studies used a titration of increasing NTA concentrations where the absorbance at both 300 nm and 800 nm increased before a plateau was reached indicating all Cu²⁺ was complexed with NTA (1:1).

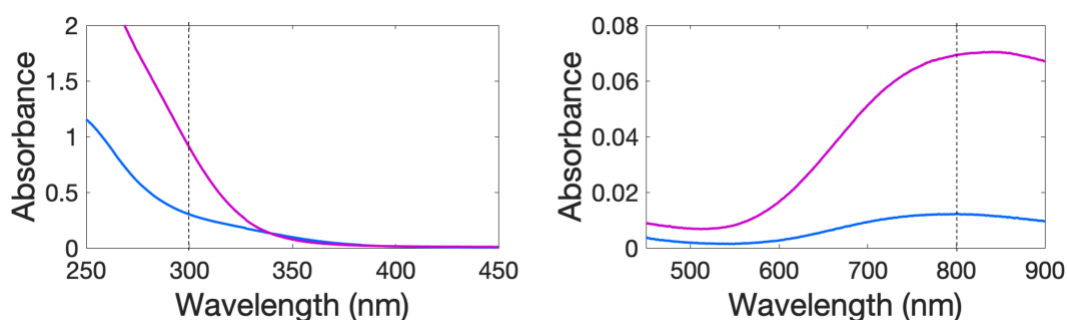


Figure 5.11 UV-Vis spectra of CuCl₂ (blue) and Cu²⁺-NTA (purple) in buffer at 300 nm (left) and 800 nm (right).

5.5.3 Spin labelling GltPh CM7 with Cu²⁺-NTA

Once the poly-histidine tag was removed, spin labelling with Cu²⁺-NTA was employed based on published studies. Ghosh et al. found 1.5 equivalents of Cu²⁺-NTA per dHis motif to be optimal, maximizing loading efficiency whilst minimizing free Cu²⁺-NTA.¹⁹⁹ Similarly, Gamble et al. found 1 equivalent per dHis site to be optimal.¹⁹¹ For the initial studies completed and discussed here, 1 equivalent of Cu²⁺-NTA was added per dHis motif. This ratio was selected to ensure all Cu²⁺-NTA bound to the dHis motifs, and to avoid free Cu²⁺-NTA in solution. This needs to be optimized in the future by following the change in absorbance at 340 nm upon the addition of Cu²⁺-NTA to the protein using UV/Vis spectroscopy. Excess Cu²⁺ would not only perturb the line shapes in the cw-EPR spectra, but it contributes to the background signal in pulsed EPR spectroscopy which in turn reduces the sensitivity.

5.6 Low temperature cw-EPR studies of Glt_{ph} spin labelled with Cu²⁺-NTA

5.6.1 Analysis of the formation of Cu²⁺-NTA

To support the UV/Vis spectroscopic results of the formation of Cu²⁺-NTA, similar studies were conducted using LT cw-EPR. LT cw-EPR spectroscopy was conducted at 50 K for MTSL-labelled samples, however due to the faster relaxation times (T_1) of copper the temperature was reduced to 10 K.¹⁵¹ Additionally, all LT cw-EPR spectra were recorded in buffer and 30% v/v glycerol to replicate the conditions used for pulsed EPR studies of Glt_{ph}.

An initial experiment was conducted comparing CuCl₂ with Cu²⁺-NTA (formed from NTA and CuCl₂ in a 1:1 ratio), where CuCl₂ represented free Cu²⁺ in solution. The LT cw-EPR spectra yielded very different line-shapes due to the different geometries around the Cu²⁺ from the coordinated monodentate Cl⁻ ligands and tetradentate NTA (Figure 5.12). The different spectral line shapes can be quantitatively analysed using EasySpin to obtain the $g_{||}$ and $A_{||}$ values indicative of the coordination environment. Visually the $g_{||}$ and $A_{||}$ appear as four equally spaced peaks, three of which are clearly

visible in Figure 5.12 with the fourth not resolved from the peak due to resonances in the plane of the molecule. A typical Cu signal with $g_{\parallel}$ and $A_{\parallel}$ values resolved, and unresolved $g_{\perp}$ is shown in Appendix A21.

There is a clear difference in the position of the peaks between CuCl_2 with Cu^{2+} -NTA in Figure 5.12 which corresponds to different A - and g - values as shown by previous studies.^{191,199} There is also clearly no CuCl_2 present in the sample (i.e. unreacted with NTA) as this would appear superimposed on the spectral line shape, like free MTSL in a protein-bound spectrum. These results further confirm the addition of NTA to CuCl_2 in stoichiometric amounts was successful in achieving complete formation of the complex, which in turn was used as a spin-label for Glt_{ph} .

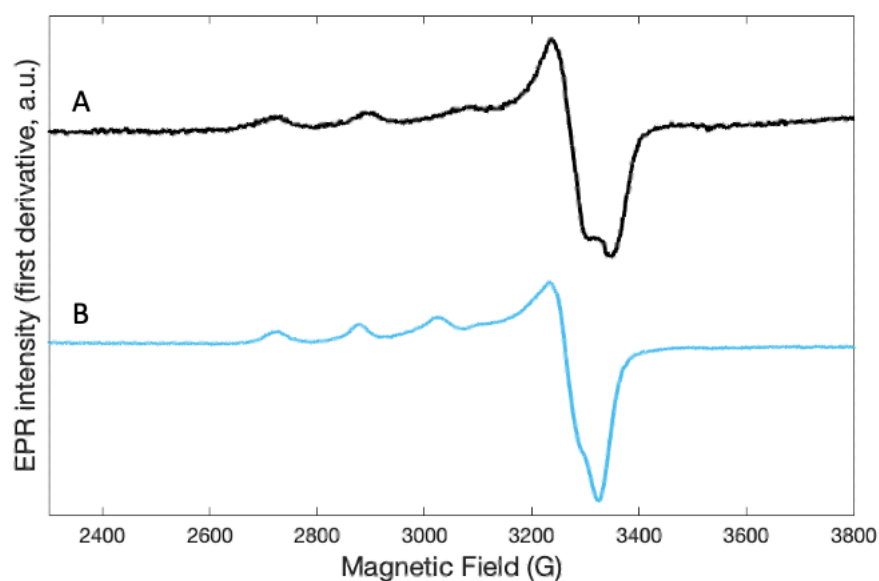


Figure 5.12 X-band LT cw-EPR spectra of A) CuCl_2 (black) and B) Cu^{2+} -NTA (blue) in buffer at 10 K. Experimental parameters: microwave frequency = 9.434 GHz, field modulation frequency = 100 kHz, modulation amplitude = 4G, microwave power = 0.9985 mW, number averages = 9, time constant = 40.96 ms, conversion time = 40.96 ms.

5.6.2 Cu^{2+} -NTA labelled Glt_{ph} CM7

LT cw-EPR was used to assess successful attachment of Cu^{2+} -NTA to the dHis motif of Glt_{ph} CM7. There was a slight shift in the $g_{\parallel}$ and $A_{\parallel}$ which indicated a change in the coordination environment (Figure 5.13). The largest change was observed for the

underlying second Cu^{2+} -NTA species which became less intense. EasySpin simulations were used to confirm the presence of multiple Cu^{2+} -NTA species and assign their g- and A- values providing quantitative values to assign any changes in the coordination environment (included in Section 5.6.3). These initial observations indicated Glt_{ph} CM7 dHis motif had successfully coordinated Cu^{2+} -NTA and was further investigated using pulsed EPR experiments presented later in this chapter.

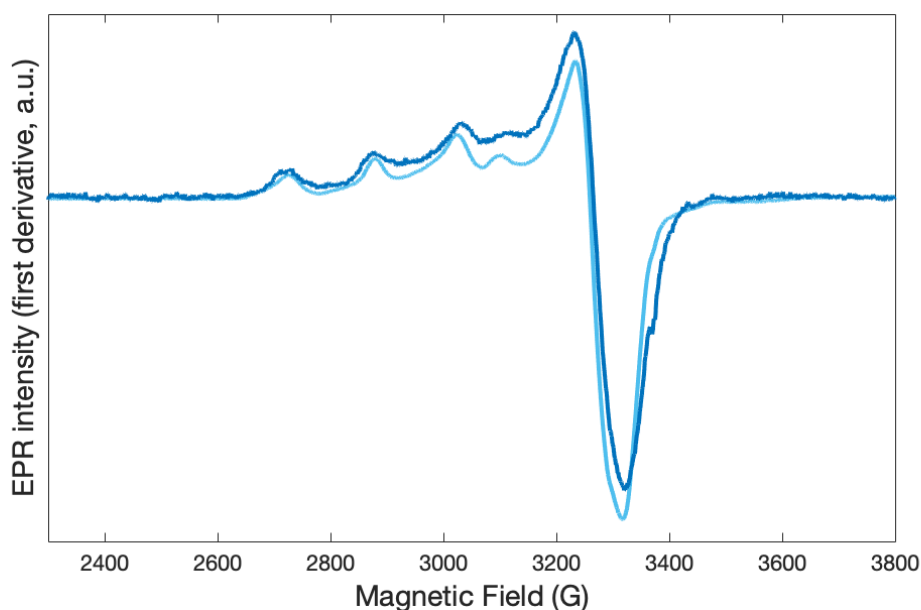


Figure 5.13 Investigating spin-labelling Glt_{ph} CM7 dHis motif with Cu^{2+} -NTA using X-band LT cw-EPR spectroscopy at 10 K. Free Cu^{2+} -NTA (blue) was recorded in buffer and when bound to Glt_{ph} CM7 (dark blue). Experimental parameters: microwave frequency = 9.434 GHz, field modulation frequency = 100 kHz, modulation amplitude = 4G, microwave power = 0.9985 mW, number averages = 9, time constant = 40.96 ms, conversion time = 40.96 ms.

5.6.3 Orthogonally labelled (MTSL and Cu^{2+} -NTA) Glt_{ph} CM7

After confirming the successful attachment of Cu^{2+} -NTA to Glt_{ph} CM7, the next step was to label Glt_{ph} CM7 with both Cu^{2+} -NTA and MTSL. Glt_{ph} CM7 was spin-labelled with MTSL during the purification process, followed by poly-histidine tag cleavage and incubation with Cu^{2+} -NTA. Figure 5.14 shows the resultant LT cw-EPR spectrum which contained the MTSL signal seen in Figure 5.9 and the Cu^{2+} -NTA species superimposed. The position of the equally spaced Cu^{2+} peaks were compared to free Cu^{2+} -NTA and both singly labelled protein samples. The results of the orthogonally

labelled sample showed the same equally spaced peak positions as Glt_{ph} CM7 with Cu²⁺-NTA. The main difference in the orthogonally labelled sample was the presence of the MTSL peak, which aligned with previously recorded Glt_{ph} CM7 MTSL spectrum confirming the presence of Cu²⁺-NTA had not impacted MTSL-binding, and vice versa with the Cu²⁺-NTA peaks. Overall, these results indicated labelling with both Cu²⁺-NTA and MTSL was successful, and the sample was tested using pulsed EPR spectroscopy.

	g_{zz}	g_{yy}	g_{xx}	A_{xx} (MHz)	A_{yy} (MHz)	A_{zz} (MHz)	Lwpp (mT)
CM7 + MTSL	2.008	2.006	2.002	13	15	99	0.7
Orthogonally labelled CM7	2.008	2.006	2.002	13	15	99	0.7

	$g_{\perp}$	$g_{\parallel}$	$A_{\perp}$	$A_{\parallel}$	Lwpp (mT)
Cu²⁺-NTA in buffer	2.068	2.292	55	450	6.2
CM7 + Cu²⁺- NTA	2.062	2.282	65	480	6.2
Orthogonally labelled CM7	2.062	2.282	65	480	6.2

Table 5.3 Best fit simulation values for LT X-band cw-EPR spectra of Glt_{ph} CM7 spin labelled with MTSL and/or Cu²⁺-NTA with reference to Cu²⁺-NTA in buffer. Results are shown in the top table for MTSL species when bound to Glt_{ph} CM7 (RMSD 0.9885) and the MTSL species in orthogonally labelled Glt_{ph} CM7 (RMSD 1.5341). The x, y, and x components of the g- and A-tensor and the linewidth (peak to peak, lwpp) are shown for each. The bottom table shows the simulation results for copper species of Cu²⁺-NTA in buffer (RMSD 6.2264), Cu²⁺-NTA labelled Glt_{ph} CM7 (RMSD 7.1538) and the Cu²⁺-NTA species in orthogonally labelled Glt_{ph} CM7 (RMSD 1.5341). The parallel and perpendicular components of the g- and A-tensor and the linewidth (peak to peak, lwpp) are shown for each. A ratio of 5 Cu²⁺-NTA: 1 MTSL species in the orthogonally labelled sample was obtained.

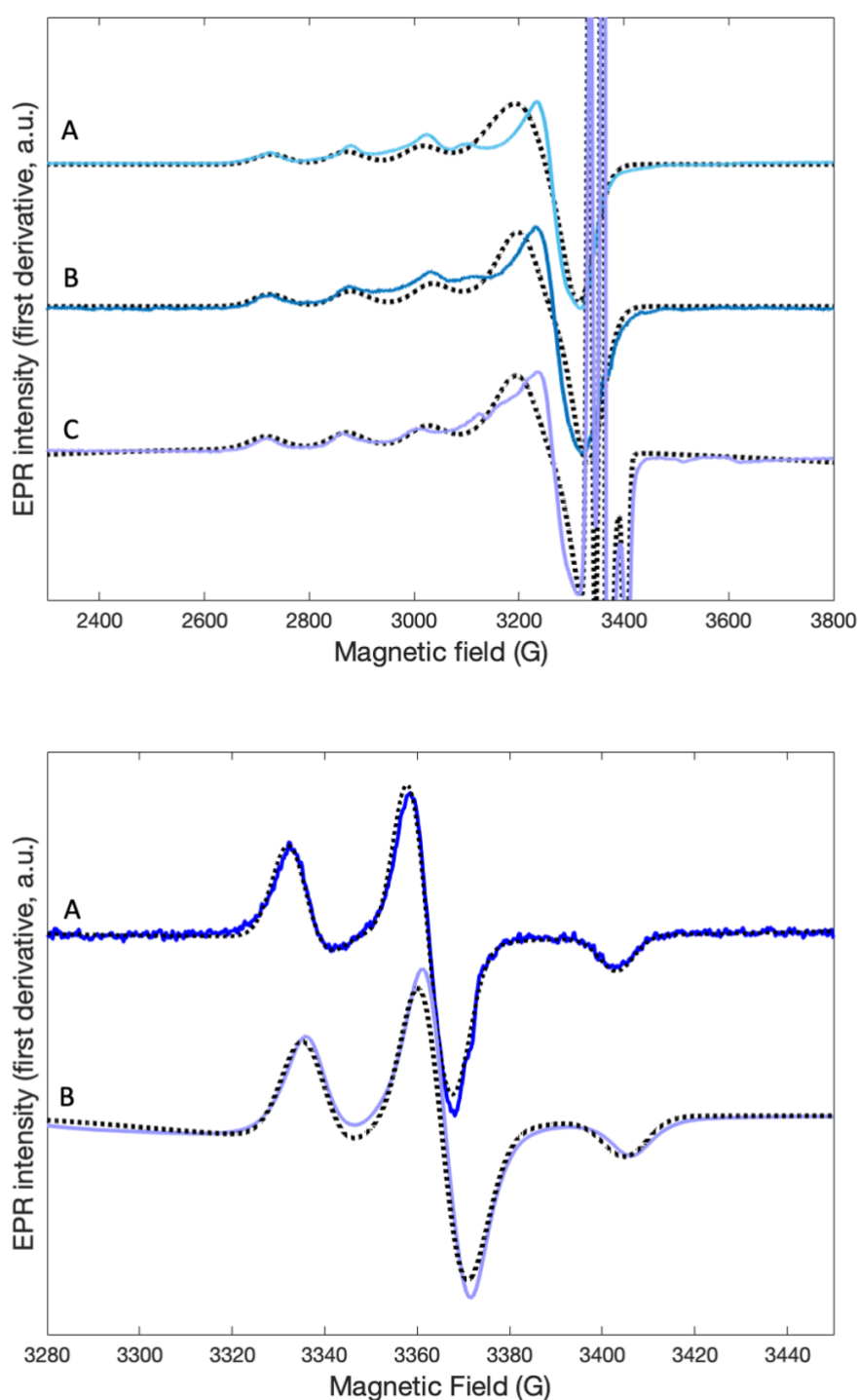


Figure 5.14 EasySpin simulations of LT cw-EPR spectra of Glt_{ph} CM7 with MTSL and/or Cu²⁺-NTA at 10 K (or 50 K for MTSL-only). Top: Expanded y-axis to show Cu features more clearly: A) Cu²⁺-NTA in buffer (blue); B) Glt_{ph} CM7 with Cu²⁺-NTA (dark blue); C) Glt_{ph} CM7 with Cu²⁺-NTA and MTSL (lilac). Bottom: The MTSL region of the spectra of A) Glt_{ph} CM7 with MTSL (bright blue) and B) orthogonally labelled Glt_{ph} CM7 with Cu²⁺-NTA and MTSL (lilac). The EasySpin simulation is shown as a black dashed line superimposed on each. Experimental parameters: microwave frequency = 9.434 GHz, field modulation frequency = 100 kHz, modulation amplitude = 4G, microwave power = 0.9985 mW, number averages = 9, time constant = 40.96 ms, conversion time = 40.96 ms.

To quantify the changes seen for i) MTSL labelled Glt_{ph} CM7, ii) Cu²⁺-NTA in buffer, iii) Cu²⁺-NTA labelled Glt_{ph} CM7 and iv) orthogonally labelled Glt_{ph} CM7, EasySpin simulations were used. MTSL species require the three component (xx, yy, zz) g- and A-values, with Cu²⁺ needing just two ($\perp$ and $\parallel$) and a larger linewidth. Simulations were completed using a single species, despite two Cu²⁺-NTA species within the LT spectral line shapes. Fitting the Cu²⁺-NTA spectra proved challenging so to characterise the change upon addition of protein, the main species was simulated, and the second underlying species discounted. The results of the simulations can be seen in Figure 5.14 and Table 5.3.

The same simulation parameters were used for fitting MTSL labelled Glt_{ph} CM7 and the MTSL species within the orthogonally labelled sample. This indicated the MTSL environment remained the same in the presence of the additional Cu²⁺-NTA label. Comparing the simulation results for Cu²⁺-NTA in buffer and when bound to the dHis motif on Glt_{ph} CM7 resulted in a shift in both the parallel and perpendicular values. This confirmed the change in coordination environment. Like the MTSL results, the parameters between Cu²⁺-NTA labelled Glt_{ph} CM7 and orthogonally labelled Glt_{ph} CM7 remained unchanged.

Spin-labelling efficiency was determined for MTSL labelled Glt_{ph} CM7 to be 81% (Table 5.2). To quantify the spin-labelling efficiency of Cu²⁺-NTA labelled Glt_{ph} CM7 the double integration values of the simulated MTSL labelled and Cu²⁺-NTA labelled spectra were calculated, giving similar double integration values (554 for MTSL and 592 for Cu²⁺-NTA). The Cu²⁺-NTA containing Glt_{ph} CM7 samples were recorded at 10 K, including orthogonally labelled. At this temperature the MTSL signal (in the orthogonally labelled sample) is saturated, but not saturated for Cu²⁺-NTA. The saturation of the MTSL signal means the electrons are relaxing slowly and lose the population difference, causing a suppressed EPR signal intensity. Under saturating conditions of MTSL, the double integration values indicate MTSL is not present in excess over Cu²⁺-NTA so under-labelling has not occurred. The 5 Cu²⁺-NTA per MTSL determined in the EasySpin simulation of orthogonally labelled Glt_{ph} CM7 would have an artificially increased ratio of Cu²⁺-NTA (due to the saturating conditions for MTSL),

indicating there is a similar labelling efficiency for both spin labels. These results mean all distances are expected to be seen in the pulsed EPR data. To accurately calculate the Cu²⁺-NTA labelling efficiency, the spectra could be recorded at 50 K (i.e. non-saturating conditions) and the resultant spectra simulated using EasySpin. In the initial reports, there is no reported extinction coefficient for Cu²⁺-NTA bound to a dHis motif. Therefore, the intensity of an optical band (in UV/Vis spectroscopy) cannot be used to assess concentration.

5.7 Pulsed EPR spectroscopy of Cu²⁺-NTA labelled Glt_{ph} for distance determination

5.7.1 Confirming successful binding of Cu²⁺-NTA to dHis motif using pulsed EPR spectroscopy

Electron spin echo envelope modulation (ESEEM) was used to investigate the binding of Cu²⁺-NTA to the dHis motif. The initial results of LT cw-EPR indicated successful attachment of Cu²⁺-NTA to Glt_{ph} CM7 as the change in peak positions suggested a change in the coordination environment. To further confirm these results a 3-pulse ESEEM experiment was conducted (by Dr Fraser MacMillan). 3-pulse ESEEM is a pulsed EPR technique where three $\pi/2$ microwave pulses are separated by evolution times to create a Hahn echo (Figure 5.15). The echo intensity is measured as a function of time and is modulated when the time delay between pulses is varied. The time domain data is converted to frequency domain using Fourier transform, where the position of the ESEEM lines can be used to identify the types of nuclei surrounding the spin label.²⁸

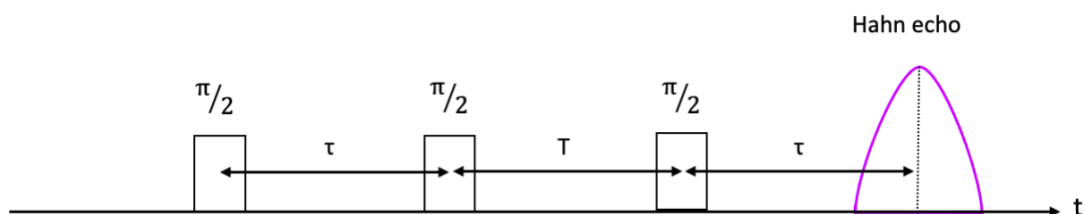


Figure 5.15 The 3-pulse ESEEM sequence - T is incremented giving rise to the stimulated echo, with τ remaining fixed. Echo intensity is measured as a function of $\tau + T$ yielding a time domain spectrum.

The ESEEM experiment was conducted using published methodology to provide comparable traces.¹⁹¹ The time domain traces of Cu²⁺-NTA in solution and Cu²⁺-NTA labelled Glt_{ph} CM7 vary significantly (Figure 5.16). Free Cu²⁺-NTA in buffer showed a simple decay with no nuclear modulations due to coupling to magnetic nuclei. This result was expected as the only nitrogen atoms present in Cu²⁺-NTA are directly coordinated and are undetected in the experiment. When bound to the dHis motif of the protein, there were clear nuclear modulations which resembled those reported by Gamble et al. deemed to be characteristic of the imidazole nitrogen coordination.¹⁹¹ These results further confirmed the successful attachment of Cu²⁺-NTA to Glt_{ph} CM7.

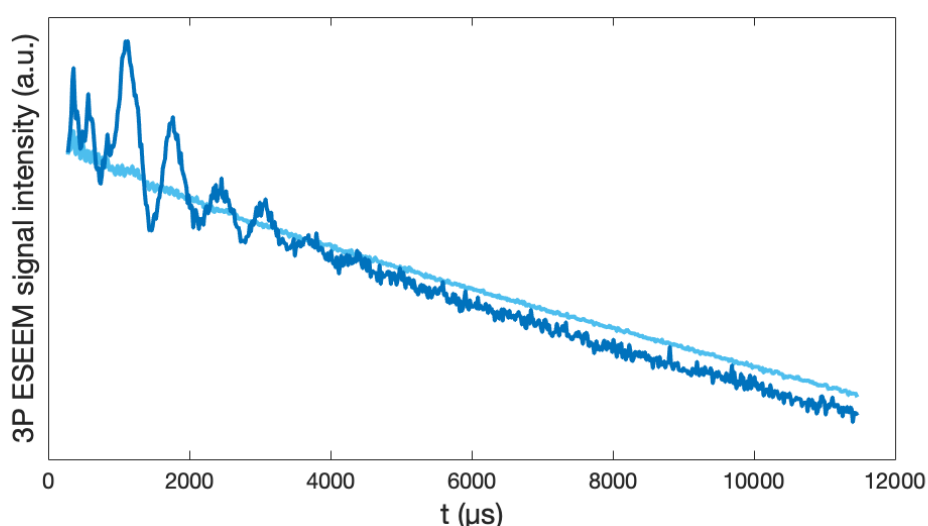


Figure 5.16 3-pulse ESEEM experiment of Cu²⁺-NTA in buffer (blue) and Cu²⁺-NTA labelled Glt_{ph} CM7 (dark blue).

5.7.2 Initial DEER studies using Cu²⁺-NTA as a label for Glt_{ph} CM7

Once Cu²⁺-NTA labelling of Glt_{ph} CM7 was deemed successful using LT cw-EPR and ESEEM, functional studies were conducted using 4-pulse DEER spectroscopy at 20 K. The 4-pulse DEER experiment was recorded for apo Glt_{ph} CM7 labelled with Cu²⁺-NTA, following the addition of Na⁺ and also substrate (L-aspartate). The experimental parameters needed to have a much wider magnetic field sweep width and a different shot repetition time as detailed in the Materials and Methods (Chapter 8). The resultant FSE was significantly different to MTSL-labelled Glt_{ph} CM7 (Figure 5.17)

resulting in the selection of different pump and detect positions for Cu²⁺-NTA labelled samples. The phase memory relaxation time determined by the T₂ experiment didn't differ significantly between the two spin-labels. The characteristic oscillations indicating the presence of deuterium (from d₈-glycerol) were present on the time evolution traces for both (Appendix A22).

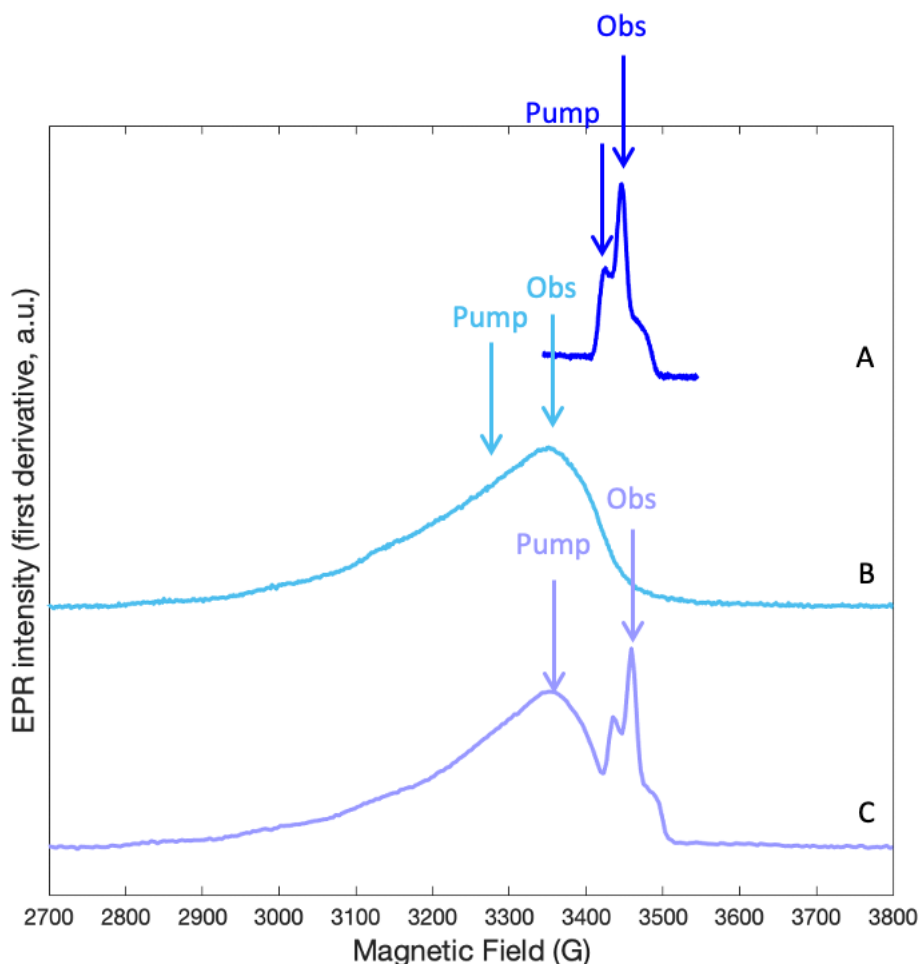


Figure 5.17 Field swept echo experiment of Glt_{ph} CM7 spin labelled with A) MTSL (dark blue), B) Cu²⁺-NTA (light blue) and C) orthogonally labelled with both (lilac). Experimental parameters are given in Chapter 8.

Following the optimization experiments, a 4-pulse DEER experiment was recorded for each sample to elucidate conformational changes during the transport cycle. The distance distributions in the three binding states were extracted from the raw experimental data using DeerNet, and the results are shown in Figure 5.18. The modulation depth for all three binding states was very small, particularly compared to MTSL-labelled Glt_{ph} CM7 in Figure 5.19: 0.5% compared to 5%.

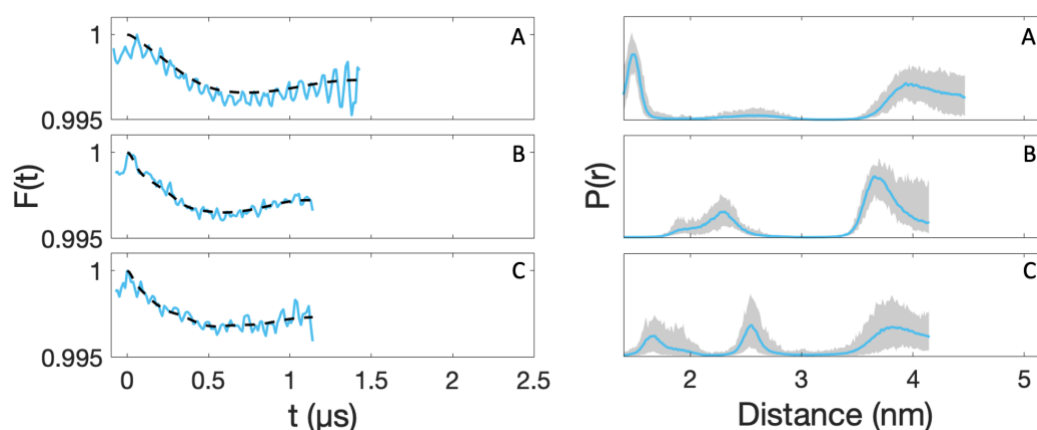


Figure 5.18 4-pulse DEER experiments of Cu²⁺-NTA labelled Glt_{ph} CM7 in detergent micelles utilising substrate and co-transported Na⁺ to trap Glt_{ph} in proposed intermediates of the transport cycle. A) apo, B) 50 mM Na⁺, and C) substrate bound (50 mM Na⁺ & 1 mM L-aspartate). The normalised background corrected time decays are shown left with their respective fittings shown as black dashed lines. The right hand traces show the calculated distance distributions using DeerNet and the 95% confidence bounds represented by the light grey areas.

All MMM predicted distances are similar for Cu²⁺-NTA labelled Glt_{ph} CM7, suggesting almost no visible changes should be observed (Table 5.1). The dHis motif where Cu²⁺-NTA binds is located on TM2 as part of the scaffold domain, which remains rigid during the transport cycle. For all structures analysed using MMM in a variety of binding states and conformations, the predicted distances were in the range 3.2–3.4 nm. The distance distributions calculated using DeerNet included distances both higher and lower than this range, but not within. The distances below 2 nm seen for apo and substrate bound Glt_{ph} CM7 are below the reliable limit for distance determination using DEER studies. Changes in distance distribution were observed between the three binding states could indicate the protein was undergoing conformational changes, however it is difficult to correlate these with specific conformations based on the MMM predictions. The low modulation depths and high noise are likely to account for the distance distributions observed. At the end of the time traces in Figure 5.18 the noise levels increase and have been interpreted as distance distributions around 4 nm. Some of the shorter distance peaks are of a similar height to the uncertainty distribution so it cannot be said with 95% confidence that the distance exists. Overall, the distances observed do not make physiological

sense when comparing them to the predicted distances of Cu²⁺-NTA labelled Glt_{ph} CM7 using MMM.

A 4-pulse DEER experiment was recorded for orthogonally labelled Glt_{ph} CM7 in an apo state. The same experimental set-up was used as Cu²⁺-NTA labelled Glt_{ph} CM7 for the FSE, T₂ and DEER experiments. The FSE experiment (Figure 5.17) showed the overlap between the MTSL and Cu²⁺-NTA signals, which aligned with the singly labelled samples. To record a 4-pulse DEER experiment which looked at both spin-labels, the pump and detect positions were selected to be in a region which encompassed both the MTSL and Cu²⁺-NTA species (Figure 5.17). The distance distribution was extracted from the raw time trace using DeerNet and compared to apo Glt_{ph} CM7 labelled with MTSL and Cu²⁺-NTA separately (Figure 5.19). The positions of the MTSL and Cu²⁺-NTA labels are shown in Figure 5.4.

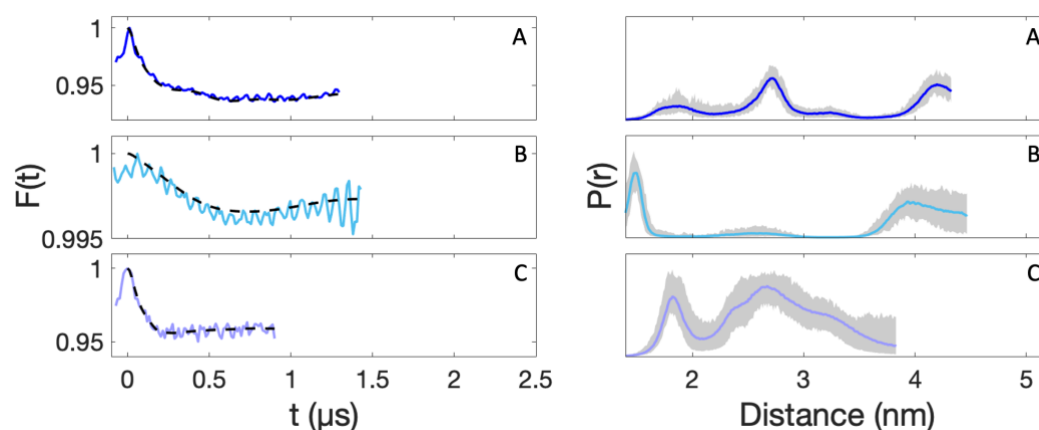


Figure 5.19 4-pulse DEER experiments of apo Glt_{ph} CM7 labelled with A) MTSL (dark blue), B) Cu²⁺-NTA (light blue) and C) orthogonally labelled with both (ilac). The normalised background corrected time decays are shown left with their respective fittings shown as black dashed lines. The right hand traces show the calculated distance distributions using DeerNet and the 95% confidence bounds represented by the light grey areas.

The distance distributions from MMM for orthogonally labelled Glt_{ph} CM7 predicted a large range of inter-spin distances. The presence of two spin-labels on each protomer of trimeric Glt_{ph} CM7 results in a large number of distance distributions both between and within protomers. All intra-protomer distances for Glt_{ph} CM7 were

<1.6 nm and do not account for any of the distances observed in Figure 5.19. MTSL labelled Glt_{ph} CM7 produced a main distance distribution at 2.7 nm thought to be an outward open conformation. This sample also had the largest modulation depth of the three spin-labelled samples at 5%. When labelled with Cu²⁺-NTA the modulation depth significantly decreased, resulting in an experimental time decay with a high level of noise. The extracted distance distributions did not correlate with any of the predicted distances by MMM. However, when labelling with both MTSL and Cu²⁺-NTA, there was a better modulation depth and correlation between the predicted and experimental distances. Predicted distances for orthogonally labelled Glt_{ph} CM7 ranged between 1.6–3.5 nm which encompasses all the distance distributions observed experimentally in Figure 5.19.

The complexity of labelling with both spin labels is clear in the distance distribution where a wide distance distribution between 2.2–3.5 nm was extracted. This wide distribution may encompass a wide range of protein conformations including inward occluded (2.5–2.8 nm), outward occluded (2.4 and 3.1 nm) and outward facing (3.4 nm). These results confirmed Glt_{ph} CM7 was successfully spin-labelled with both MTSL and Cu²⁺-NTA for EPR studies. The next step would be to complete functional studies on orthogonally labelled Glt_{ph} CM7 in the presence of Na⁺ and Na⁺-substrate to monitor changes in distance distribution during the transport cycle. The ultimate experiment is to attempt iDEER studies in an attempt to simplify these distributions by eliminating the dipolar interaction between pairs of like sites based on their relaxation times (described in Appendix A15).

6 Using STD NMR spectroscopy to determine ligand dissociation constants, K_D

The function of membrane transporter complexes is to control the movement of substrate molecules and ions from one side of the membrane to the other. Therefore, knowing physiological information such as ligand binding affinities is essential for understanding how they function. Common methods to determine binding affinities include isothermal titration calorimetry (ITC),^{128,200,201} surface plasmon resonance (SPR),^{179,202} fluorescence based assays^{128,129} and uptake experiments.^{129,182} Saturation transfer difference nuclear magnetic resonance (STD NMR) spectroscopy is an alternative method to these approaches. It avoids addition of an exogenous label and the intricate buffer matching between the protein and ligand samples in ITC which has added difficulty with detergent solubilised samples. The use of wild type (WT) protein also meant results were not affected by amino acid substitutions.

Recent studies within the laboratory worked to develop the limited application of STD-NMR spectroscopy to the Glutamate transporter from *Pyrococcus horikoshii*, Glt_{Ph}. Here the dissociation constants (K_D) for both L-aspartate and L-glutamate were successfully determined for Glt_{Ph} in both detergent micelles and proteoliposomes. In a competition titration, L-aspartate did not fully displace L-glutamate bound to Glt_{Ph} in a proteoliposome environment. The calculated Hill coefficient revealed negative cooperativity where binding of substrate to one binding site makes binding of another less likely. This indicated that in a proteoliposome Glt_{Ph} has an interaction between protomers, and transport (not just binding) occurs. This contrasts to Glt_{Ph} in detergent micelles where aspartate fully displaced glutamate. These findings were significant because the cooperativity observed between the protomers of the trimeric protein had not been described in literature previously.¹³³ This chapter describes the attempt to apply this technique and methodology to the closely related Glutamate transporter, Glt_{Tk}. With a 77% sequence identity between Glt_{Tk} and Glt_{Ph},¹⁰⁹ and the highly conserved binding sites, these experiments were expected to allow determination of the dissociation constants for Glt_{Tk} with L-glutamate and L-aspartate, in addition to observing the cooperativity in a second similar system to

investigate whether this behaviour could apply to the wider glutamate transporter family. Published dissociation constants for Glt_{TK} with L-aspartate are in the low nanomolar range, similar to those of Glt_{ph}. However, there are no dissociation constants for Glt_{TK} with L-glutamate in literature. The published dissociation constants for Glt_{ph} and Glt_{TK} are shown in Appendix A1 with the technique and sodium ion concentration used to calculate them. This chapter will also investigate the effect of sodium ion concentration on the STD NMR results of WT Glt_{ph}, as well as experiments using a Glt_{ph} cysteine variant with and without spin label (MTSL).

6.1 WT Glt_{TK} STD NMR studies

6.1.1 WT Glt_{TK} STD-NMR direct titration with L-glutamate

An initial experiment was first conducted to confirm that an STD signal was observable for Glt_{TK} and L-glutamate, particularly given the lack of published estimates of dissociation constants. The following control experiments were then recorded: a) buffer and L-glutamate in the absence of Glt_{TK}, b) buffer and L-aspartate in the absence of Glt_{TK} and c) WT Glt_{TK} in KCl buffer with L-glutamate. These experiments were used to prove the origin of saturation observed was from binding of the ligand (L-glutamate or L-aspartate) to Glt_{TK} and not from detergent. Since Glt_{TK} is a sodium symporter, control c) also highlighted the importance of sodium ions for the binding and transport of substrate molecules as no STD effect was observed in the absence of sodium ions.

For STD experiments, an on-resonance (saturated) spectrum at 0.6 ppm was recorded, in addition to an off-resonance (reference) spectrum at 40 ppm. The resonance for the reference is away from both protein and ligand signals. In the on-resonance experiment, selective saturation of the aliphatic side chains of the protein occurs. When a ligand molecule binds to the saturated protein, magnetisation transfers to the bound ligand. The saturated ligand dissociates from the protein into solution and is detected as an on-resonance signal before the signal decays by longitudinal relaxation. The STD spectrum is the difference between the saturated

and reference spectra, therefore only showing peaks from ligand molecules that have directly interacted with the saturated protein. For the aforementioned control experiments, the STD spectra showed no ligand peaks (Figure 6.1) meaning any STD effects which were observed in the titration experiments were a result of direct interaction of the ligand binding to Glt_{TK}.

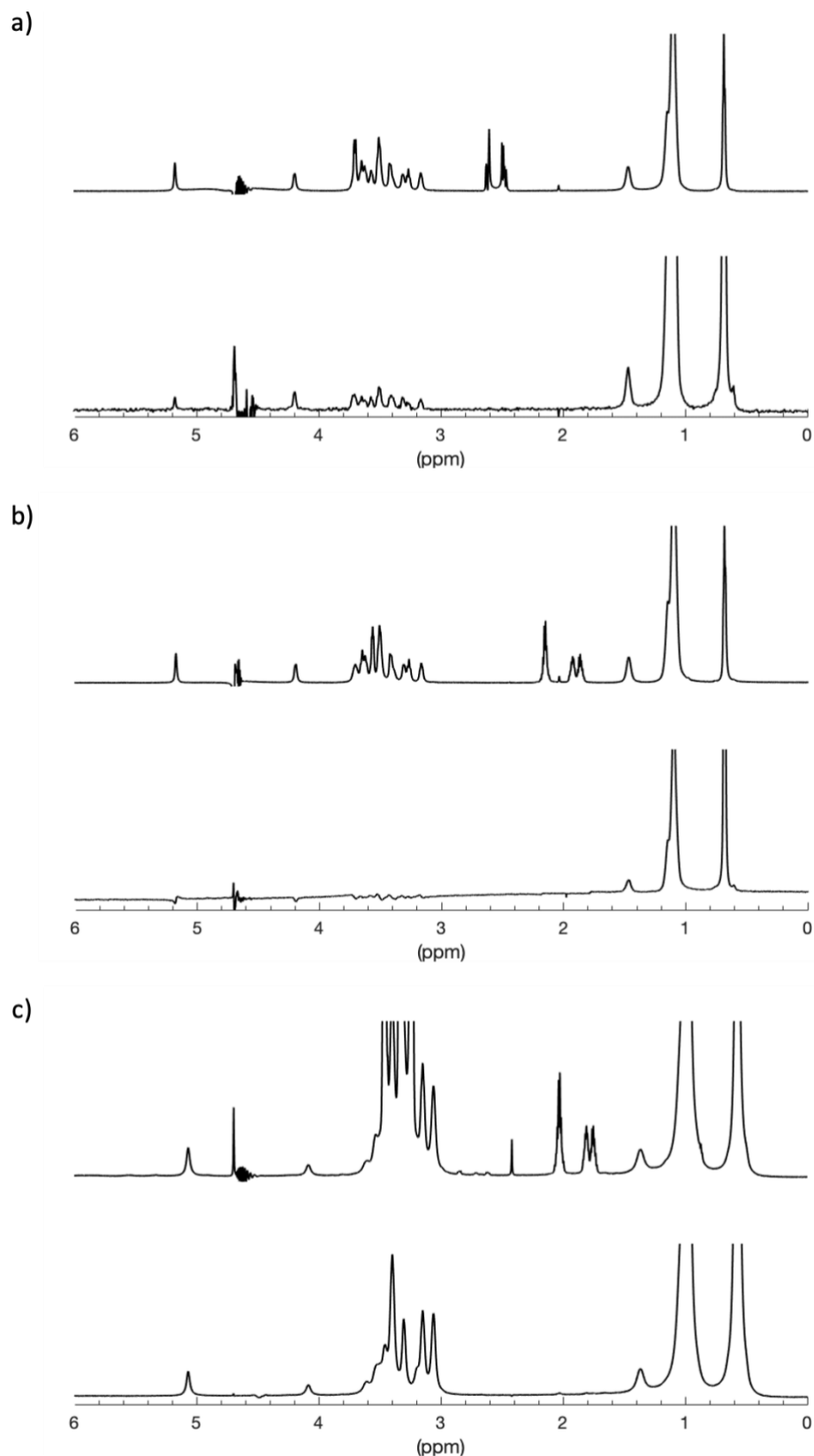


Figure 6.1 ^1H NMR spectra recorded as control experiments with 1D ^1H reference spectra (i.e. on-resonance) shown top, and STD spectra below for a) L-aspartate (1 mM) in detergent buffer b) L-glutamate (1 mM) in detergent buffer and c) WT Glt_{Tk} (18 μM) in KCl (100 mM) buffer with 0.5 mM L-glutamate. Spectra were recorded at 800 MHz, 278 K with a saturation time of 2 s. Spectra have been scaled to make the STD effect clear.

A test experiment of WT Glt_{TK} with excess (1 mM) L-glutamate was recorded to confirm an STD effect could be observed under the selected conditions and therefore the titration experiments to quantitatively determine a K_D value could commence. These experiments followed the published protocol whereby L-glutamate was added to WT Glt_{TK} over a range of ligand concentrations. If the K_D was known, the range would be selected to span either side to eventually reach a point of saturation with excess ligand. However, without this information the ligand concentrations were selected based on those used for Glt_{ph} i.e. starting at 50 µM L-glutamate and increasing until saturation was observed. For each addition of ligand an STD spectrum was calculated, and the STD effect was measured (Figure 6.2). L-glutamate had two main signals in the ¹H NMR spectrum which could be selected to measure the STD effect: the γ protons just above 2 ppm and the β protons at 1.8 ppm. For consistency, the γ protons were selected to measure the STD effect for all L-glutamate titrations.

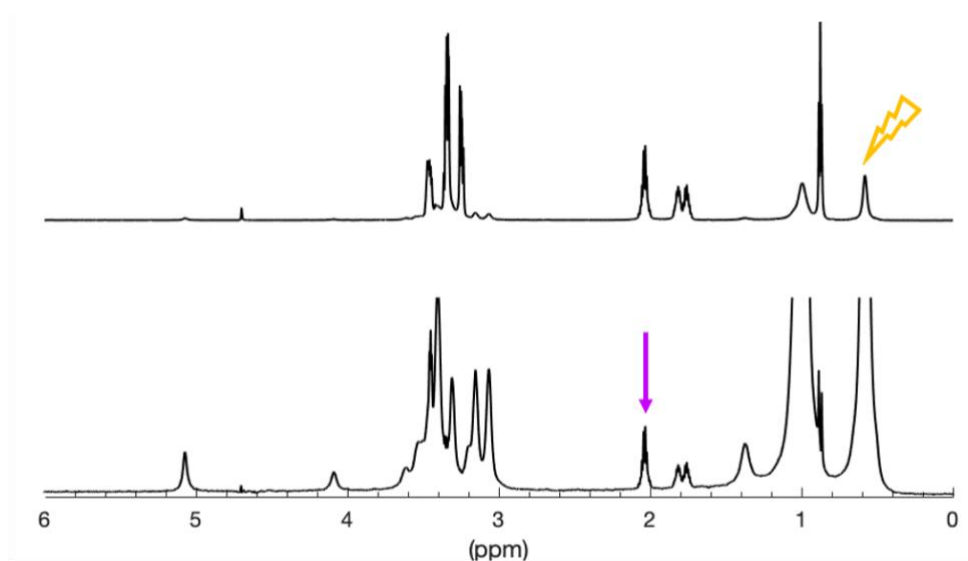


Figure 6.2 ¹H NMR spectra of detergent solubilised WT Glt_{TK} (18 µM) in buffer containing 100 mM NaCl following incubation with 5 mM L-glutamate. Top: off-resonance reference spectrum. Yellow lightning bolt at 0.6 ppm represents the point of saturation used in the on-resonance experiment. Bottom: resultant STD spectrum where γ protons of L-glutamate were used to calculate an STD effect. Spectra were recorded at 800 MHz, 278 K with a saturation time of 2 s. Spectra have been scaled to make the STD effect clear.

The STD effect was used to calculate an STD amplification factor (STD-AF, Equation 6.1) which accounts for protein concentration [P]_{total}, ligand concentration [L]_{total}, and

the ligand signal intensity in the STD spectrum where I_0 is the ligand signal intensity in the reference NMR spectrum and I_{sat} is the ligand signal intensity in the saturated spectrum.

$$STD - AF = \frac{I_0 - I_{sat}}{I_0} \times \frac{[L]_{total}}{[P]_{total}} \quad 6.1$$

The STD-AF was calculated for each titre and plotted against $[L]_{total}$, and the resultant curve was fitted using the MATLAB curve fitting toolbox with Equation 6.2. The result gave an STD curve from which the maximum STD-AF (α_{STD}) and K_D were extracted.

$$STD - AF = \frac{\alpha_{STD}[L]}{K_D + [L]} \quad 6.2$$

After the addition of excess substrate, binding curves should reach a plateau to indicate full saturation has been achieved. For WT Glt_{Tk} with L-glutamate, this was never observed even with a vast excess of substrate (15 mM). Figure 6.3 shows the binding curve obtained for the titration of Glt_{Tk} with L-glutamate at concentrations between 50 μ M to 10 mM from which a K_D of 11 mM was calculated. Further experiments were conducted with a) ligand concentrations ranging between 5–15 mM to investigate the 7.5 mM datapoint in Figure 6.3 (in case this was the start of a new saturation curve), and b) using a lower protein concentration to check if a smaller STD effect is observed giving a comparable STD amplification factor. Both of these additional experiments gave similar results to the STD curve presented below. Therefore, the K_D value calculated based on the STD curve in Figure 6.3 (11 mM) raises questions surrounding the success of this experiment and validity of this result, particularly when compared to the K_D of WT Glt_{ph} with L-glutamate determined using this same methodology (414 μ M).¹³³ The presence of the STD signal, and the changes observed upon different ligand concentrations, means that ligand molecules are binding (in some capacity) to the protein to receive saturation. However, the binding could be non-specific. In Glt_{ph}, L-aspartate and L-glutamate bind

to the same site and with the high sequence similarity, particularly in the binding sites, one would assume a similar behaviour would be observed for Glt_{TK}.

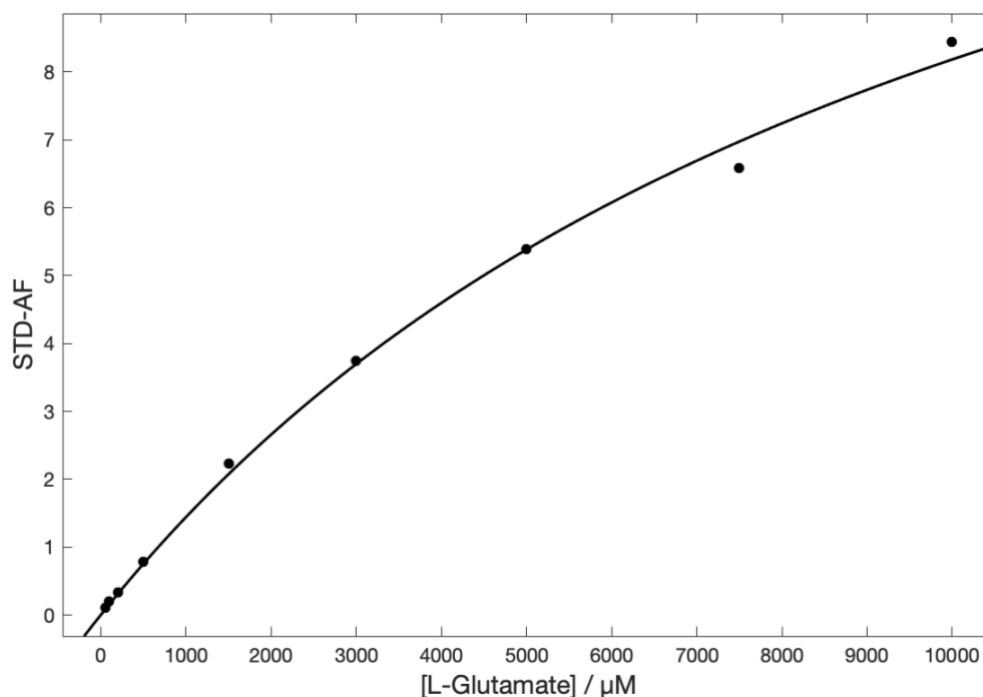


Figure 6.3 A plot of the STD-AF against L-glutamate concentration for WT Glt_{TK} (18 μM) with 100 mM NaCl. The curve was fitted using the MATLAB curve fitting toolbox which was used to determine a K_D value of 11 mM with an R^2 of 0.9967 and $\alpha_{\text{STD}} = 17.06$.

6.1.2 WT Glt_{TK} STD-NMR competition titration with L-aspartate

Unlike L-glutamate, there are several published K_D values for Glt_{TK} with L-aspartate which is likely to be due to the greater biological relevance of this ligand for the archaeal homologues of the glutamate transporters. These range between 62–1040 nM (using NaCl concentrations between 75–500 mM)¹¹³ compared to those for Glt_{Ph} with published values between 2–42 nM (using NaCl concentrations between 100–200 mM).^{111,112,124,126,128,129,133,182} As NaCl concentration is decreased, K_D values increase (Appendix A1). Initially a competition titration was used to determine a K_D value for Glt_{TK} with L-aspartate to test if the ligand and protein are in fast exchange. This type of titration was necessary due to the high affinity of L-aspartate, making observation of the ligand binding and dissociating unlikely. Increasing concentrations of L-aspartate were titrated into WT Glt_{TK} with a fixed concentration of L-glutamate present. As L-aspartate is added, it displaces the L-glutamate (which has a lower

affinity) and the decrease in L-glutamate signal is recorded as the STD effect (Figure 6.4). The concentration of L-glutamate is selected so it is high enough to have an observable STD effect, whilst being in excess. Although the L-glutamate STD curve did not reach saturation and plateau, the concentrations selected (either 3 or 5 mM L-glutamate) were 150 or 250 times greater than the protein concentration to fit these criteria.

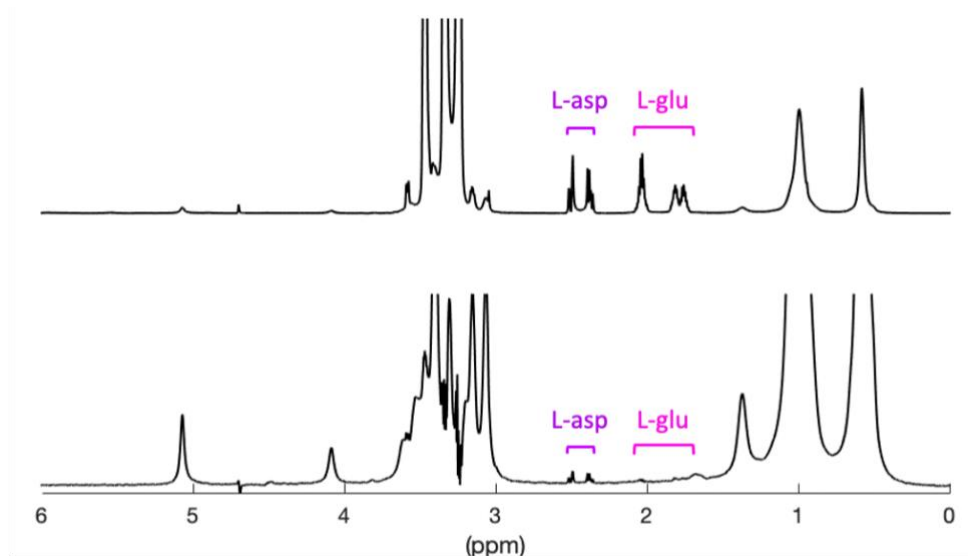


Figure 6.4 ^1H NMR spectra of detergent solubilised WT Glt_{TK} (25 μM) in buffer containing 100 mM NaCl following incubation with 3 mM L-glutamate (L-glu) and 3 mM L-aspartate (L-asp). Top: off-resonance reference spectrum. Bottom: resultant STD spectrum where γ protons of L-glutamate were used to calculate an STD effect for the competition titration. Due to the high concentration of aspartate in these spectra, the STD effect was also recorded for aspartate. Spectra were recorded at 800 MHz, 278 K with a saturation time of 2 s. Spectra have been scaled to make the STD effect clear.

An initial experiment with no L-aspartate present was first recorded so the STD effect measured could be assigned as 100%. The STD effects for L-aspartate additions were measured relative to this and the % bound (or normalised STD effect) values were plotted against L-aspartate concentration. The MATLAB curve fitting tool was used with Equation 6.3, where A is a proportionality constant, $K_{D_{glu}}$ is the measured dissociation constant for L-glutamate ($1100 \times 10^4 \mu\text{M}$), L_0 is the fixed concentration of L-glutamate used (3000 or 5000 μM), I_0 is the concentration of L-aspartate added and $K_{D_{asp}}$ is the dissociation constant of L-aspartate to be determined.

$$\% \text{ bound} = -A \times \frac{\left[\frac{K_{D_{glu}}}{L_0} \right] \left[\frac{I_0}{K_{D_{asp}}} \right]}{1 + \left[\frac{K_{D_{glu}}}{L_0} \right] \left[1 + \left(\frac{I_0}{K_{D_{asp}}} \right) \right]} + 100$$

6.3

Figure 6.5 shows the binding curve obtained for WT Glt_{TK} titrated with L-aspartate in the presence of 5 mM L-glutamate. A K_D value of 17 nM was calculated which is lower than the range of published values for WT Glt_{TK}. It is worth noting that this was determined using the K_D value of L-glutamate from the direct titration for which the validity is uncertain. In addition, the resultant STD curve showed L-aspartate only managed to displace less than 10% of the bound L-glutamate. This is an unexpected result due to L-aspartate being the preferred substrate for the archaeal homologues, and the lack of published literature for L-glutamate. In the comparative STD NMR experiments of WT Glt_{ph} almost full displacement was achieved in detergent and in proteoliposomes 67% L-glutamate was displaced (exhibiting the cooperative behaviour). This adds further uncertainty to the results of these STD NMR titration experiments for WT Glt_{TK}.

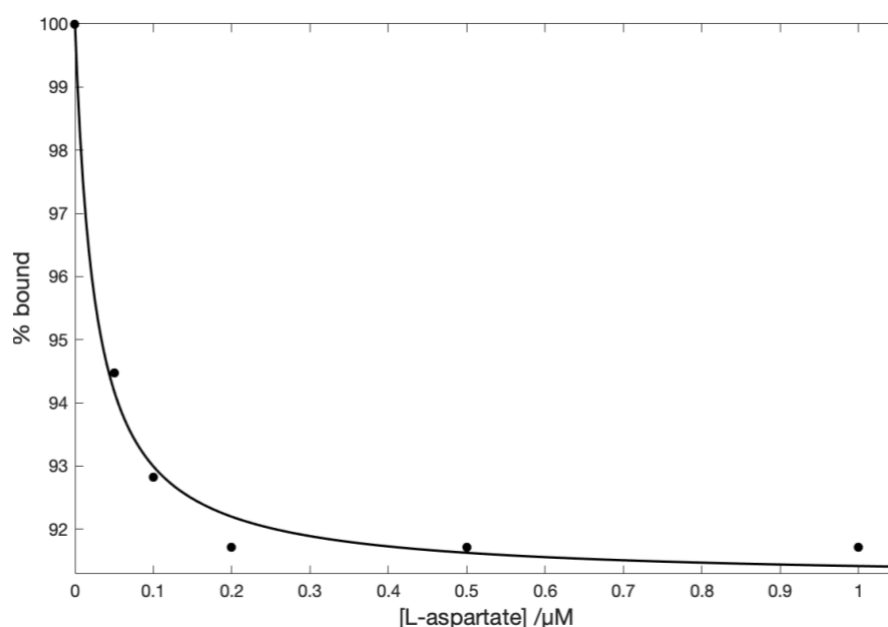


Figure 6.5 A plot of the % bound (normalised STD effect) against L-aspartate concentration for the competition titration of WT Glt_{TK} (16 μM) in the presence of 5 mM L-glutamate and 100 mM NaCl. The curve was fitted using the MATLAB curve fitting toolbox which was used to determine a K_D value of 17 nM with an R² of 0.9915, and A = 8.805.

The published K_D value of Glt_{ph} with L-glutamate determined using STD NMR experiments was 414 μ M.¹³³ Glt_{ph} and Glt_{Tk} have highly conserved binding sites for both substrate and Na⁺ ions, so similar binding affinities would be expected. Given the uncertainty in the determination of the L-glutamate K_D value with Glt_{Tk}, the K_D value of Glt_{ph} with L-glutamate was inserted into Equation 6.3. This calculated a K_D value of 1.962 nM for L-aspartate, which is even smaller than the 17 nM value determined previously. In comparison, the K_D values for L-aspartate with Glt_{ph} using STD-NMR spectroscopy were 42 nM and 13 nM for detergent micelles and liposomes respectively, with ITC showing the K_D value for Glt_{Tk} with L-aspartate is higher than Glt_{ph}.^{111–113,133}

An additional competition titration was conducted using higher concentrations of L-aspartate. Ratios of L-glutamate:L-aspartate of 1:0.5; 1:1, 1:2 and 1:4 were used. This significantly contrasts to the concentrations of aspartate used previously (up to 1 μ M). The idea of this experiment was to see if an STD effect could still be observed at higher L-aspartate concentrations. Interestingly, in the STD spectrum there were observable peaks for both L-aspartate and L-glutamate, so it was possible to measure the STD effect of both L-glutamate (as a reporter ligand) and L-aspartate directly, with the latter signal being clearer (as seen in Figure 6.4). As L-aspartate concentration increased, the STD effect of L-glutamate increased, and the STD effect of L-aspartate decreased. When using lower aspartate concentrations in the previous competition titration, as the L-aspartate concentration increased the observed STD effect of the L-glutamate decreased. This supports the uncertainty in the competition titration results.

L-glutamate and L-aspartate have been shown to bind to the same site in Glt_{ph}, which is highly conserved in Glt_{Tk}. Therefore, a similar behaviour in the binding events and STD NMR results was expected. The direct titration using L-glutamate did not reach a point of saturation, giving an initial indication that non-specific binding could be occurring. In the competition titration, to determine a K_D value for L-aspartate only 8% of the L-glutamate was displaced meaning 92% was still bound to the protein. Non-specific binding would explain why a much smaller proportion is displaced

compared to the 67% observed for Glt_{Ph}. This could be further investigated using epitope mapping or DEEP STD NMR (Section 2.5).

6.1.3 WT Glt_{Tk} STD-NMR direct titration with L-aspartate

Due to the observable STD effect for L-aspartate in the competition titration, a direct titration was attempted despite the low nM range K_D values. This followed the same procedure used for L-glutamate, using L-aspartate concentrations ranging from 0.1–5 mM. The STD effect was calculated using the β -protons at 2.4 and 2.5 ppm. The calculated STD-AF was plotted against L-aspartate concentration where the relationship observed was unclear (Figure 6.6). Therefore, a K_D value was not calculated for WT Glt_{Tk} with L-aspartate using the direct titration. These results could indicate non-specific binding was occurring due to the observable STD effect but lack of saturation and plateau.

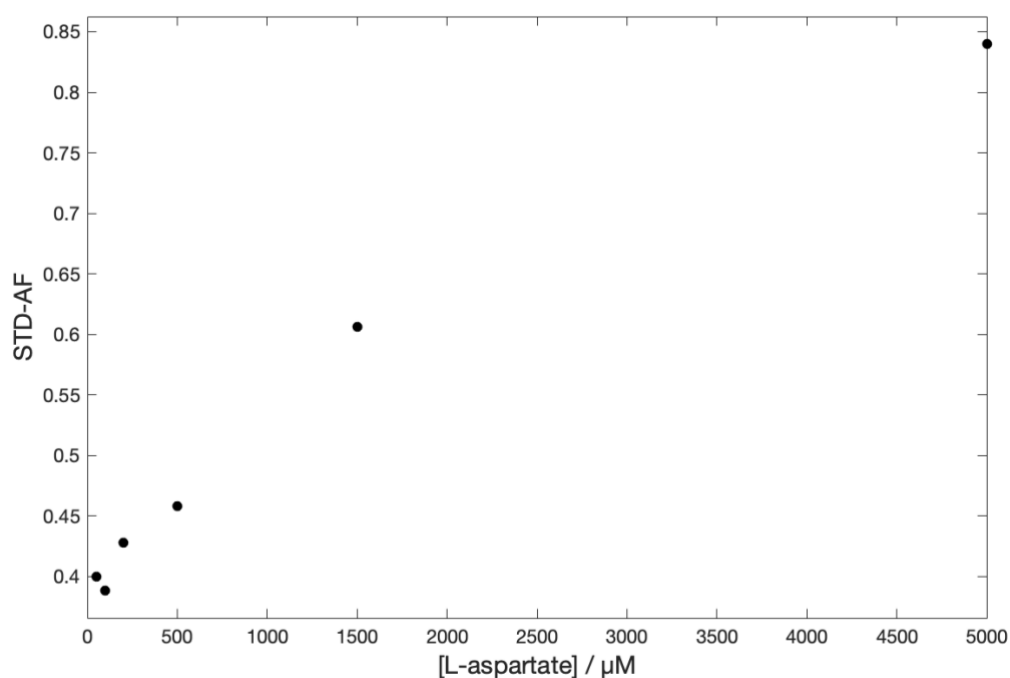


Figure 6.6 A plot of the STD-AF against L-aspartate concentration for WT Glt_{Tk} (25 μM) with 100 mM NaCl. A weak linear relationship was observed from which a K_D was not determined.

6.2 Glt_{ph} STD NMR studies

6.2.1 WT Glt_{ph} STD-NMR titration with L-glutamate at varying sodium ion concentrations

As a control-check of the STD experimental set up used for WT Glt_{rk} studies, a replication of the published STD NMR titration of WT Glt_{ph} with L-glutamate was conducted (Figure 6.7). The calculated K_D obtained was 670 μM which is within the expected range. This confirmed the WT Glt_{rk} results were not a result of experimental set-up or sample preparation.

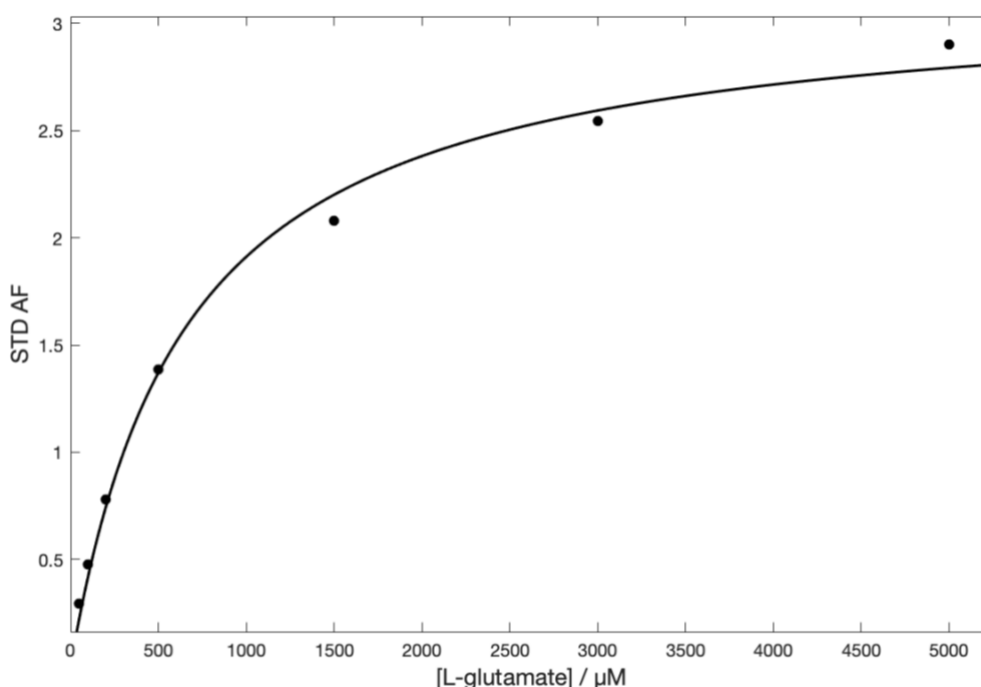


Figure 6.7 A plot of the STD-AF against L-glutamate concentration for WT Glt_{ph} (20 μM) at 100 mM NaCl. The curves was fitted using the MATLAB curve fitting toolbox to determine a K_D value of 651 μM ($R^2 = 0.9939$, $\alpha_{\text{STD}} = 3.157$).

6.2.2 STD-NMR titration with L-glutamate using Glt_{ph} C312S A71C with and without spin label

In addition to WT, Glt_{ph} C321S A72C was also studied using STD NMR for K_D determination. Glt_{ph} C321A A71C was designed for EPR spectroscopic studies with the native cysteine residue being removed, and an additional cysteine introduced for spin-label attachment. The purpose of this study was to investigate the effect of the

presence of a paramagnetic centre (spin-label, MTSL) on the STD effect and K_D . STD NMR of Glt_{ph} C321A A71C yielded a K_D value of 414 μ M and when spin-labelled a K_D value of 611 μ M which are comparable to that of WT Glt_{ph}. This confirms that, for this specific variant, mutagenesis and the presence of MTSL hasn't affected the ligand binding affinity. However, it is evident in the STD NMR curve the signal for WT is stronger than that the cysteine variant which could be a result of mutagenesis (Figure 6.8). The results of the spin-labelled experiment are likely to vary depending on the proximity of the spin-label to the binding site where L-glutamate receives saturation, therefore additional variants may need to be designed and tested. If Glt_{tk} STD NMR provided more promising results, the 6 variants available and studied in this thesis would have proven interesting due to the varying proximity of the cysteine residues to the binding site(s). But initial results of Glt_{ph} C321A A71C showed the presence of MTSL had no effect on the K_D .

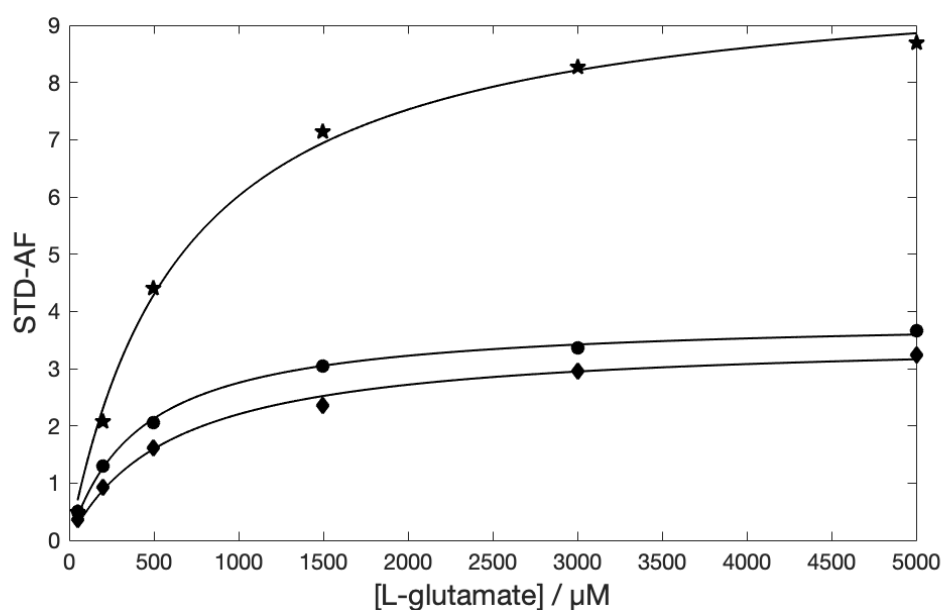


Figure 6.8 A plot of the STD-AF against L-glutamate concentration for WT Glt_{ph} (stars, 22 μ M), Glt_{ph} C321A A71C (circles, 23 μ M) and Glt_{ph} C321A A71C + MTSL (diamonds, 18 μ M) with 100 mM NaCl. The curves were fitted using the MATLAB curve fitting toolbox which was used to determine K_D values of 670 μ M ($R^2 = 0.9970$, $\alpha_{STD} = 10.05$), 414 μ M ($R^2 = 0.9971$, $\alpha_{STD} = 3.894$) and 611 μ M ($R^2 = 0.9932$, $\alpha_{STD} = 4.913$) respectively.

7 Discussion

As the most abundant neurotransmitter in the central nervous system, glutamate levels must be tightly regulated. The excitatory amino acid transporters (EAATs) are responsible for regulating the uptake of glutamate from the synaptic cleft, preventing excitotoxicity and terminate signal transmission.^{69,70} Dysfunction of these transporters are linked to various neurological disorders such as Alzheimer's disease, schizophrenia and epilepsy.^{79–81} The glutamate transporters from *Thermococcus kodakarensis*, Glt_{Tk} and *Pyrococcus horikoshii*, Glt_{ph} are archaeal homologues used as model systems for the EAAT's. The magnetic resonance studies described in this thesis worked to further elucidate the proposed alternating access mechanism of substrate transport (Section 1.5.5). This included the first EPR studies of Glt_{Tk} in various membrane mimetics, and first application of using Cu²⁺-NTA to spin-label Glt_{ph} to achieve narrower distance distributions.

7.1 Studying the conformational changes of Glt_{Tk} using EPR spectroscopy

Chapter 3 described SDSL of the six Glt_{Tk} variants studied in this thesis, following cw-EPR spectroscopy upon addition of substrate or inhibitor with co-transporter Na⁺ ions. The recorded spectra were simulated using the EasySpin software package to characterise any changes in line shape observed. These results were further explored using DEER spectroscopy to correlate changes in extracted distance distributions with proposed conformational changes during the transport cycle. Distance distributions were extracted from experimental time traces using DEER analysis software and compared to *in silico* predictions based on static crystal and cryo-EM structures. Overall results confirmed a mixture of conformations were present for each binding state. The proposed alternating access mechanism cycles between inward and outward facing conformations via a series of intermediate and occluded conformations; a range of which were observed based on rotational correlation time, τ_c , and DEER distance distributions.

WT Glt_{TK} was investigated as a control study, with the absence of native cysteine residues inhibiting the formation of a disulfide bond for attachment of spin label (MTSL). However, cw-EPR spectra showed the typical nitroxide signal of three lines, showing MTSL was present in the sample. This was concluded to be non-specific labelling and attachment of MTSL to the protein due to the absence of well-defined inter-spin distances in the analysed DEER traces, and parameters extracted from simulation of cw-EPR spectra. In each of the six Glt_{TK} variants cw-EPR spectra, residual MTSL was present in the spectral line shapes and quantified by double integration of spectra to be <6% of the total spin population in all samples using EasySpin. Spin labelling on the column was found to be the best strategy to minimise residual MTSL in the samples. The largest amount was observed for Glt_{TK} M314C which was predicted to have the lowest labelling efficiency. Similarly, the LT cw-EPR spectra of all Glt_{TK} variants indicated any changes in spectral line shape at RT were a result of τ_c , which was correlated with *in silico* predictions for different conformations. The extent of the line shape changes in RT spectra varied between variants.

A comparison between four different membrane mimetics was made: detergent micelles, proteoliposomes, SMALPs and nanodiscs. Detergent micelles proved to give the highest modulation depth in DEER traces which represented the largest number of coupled spins. Achieving high sample concentration and spin-spin relaxation times was limited in proteoliposomes, resulting in poorer signal to noise than in detergent micelles and truncated distance distributions. Further research into the specific liposome composition and size for Glt_{TK} DEER studies is needed to achieve optimal results. Previous Glt_{ph} DEER studies found more conformations were observed in a liposome environment compared to detergent micelles. This was not clear in the Glt_{TK} studies due to the level of uncertainty in some of the distance distributions which may result from high noise and therefore artificial interpretation as an oscillation. The EPR studies of Glt_{TK} S281C also represent the first EPR studies of a glutamate transporter in SMALPs and nanodiscs. SMALPs are a relatively new technique which are still being optimised, but it is arguably one of the most promising environments to study membrane proteins due to the presence of native lipids.

Residue S281 in the Glt_{TK} sequence is part of a highly conserved triple serine motif located on helical hairpin 1 (HP1). HP1 is involved in the substrate binding site and dispatch of Na⁺ to the ion binding sites. This was the most informative variant in terms of distance distributions and changes in spectral line shape using RT cw-EPR spectroscopy. However, transport assays and ITC measurements completed by collaborators at the University of Groningen (refer to Appendix A2) revealed this variant had the lowest activity of all the Glt_{TK} variants indicating inefficient transport. The ITC experiment revealed comparable K_D values to WT to confirm binding of substrate. Tests of MTSL-labelled and unlabelled Glt_{TK} S281C also revealed the presence of MTSL does not hinder the binding affinity, or transport assay results. Recent cryo-EM structures revealed asymmetry for homotrimeric Glt_{TK}, where only one of the five structures (in various binding states) had all three protomers in the same conformation when substrate bound. This was observed experimentally for Glt_{TK} S281C where apo, Na⁺-bound, and inhibitor bound protein yielded a mixture of distance distributions corresponding to inward and outward facing states. Aspartate bound Glt_{TK} S281C showed a single distance distribution corresponding to an outward occluded state based on *in silico* predictions. The distance distributions obtained using DEER studies closely correlated to *in silico* predictions in MMM using cryo-EM structures, corroborating the published structures. Spectral fitting using EasySpin revealed each binding state to contain two different correlation times for rotational motion of the spin label, which was interpreted as two protein conformations. Glt_{TK} and Glt_{Ph} have been solved in different conformations for each binding state, so these results align with the presence of multiple conformations in one binding condition. This is reflected in the proposed mechanism previously presented in Figure 1.17.

Apo Glt_{TK} S281C in detergent micelles revealed a dominant inward open conformation, and additional outward occluded state in both spectral simulations and distance distributions. The outward open state became the dominant species in spectral simulations on addition of Na⁺ with the presence of an inward open state, which again corresponded with distance distributions. Interestingly Na⁺-substrate bound Glt_{TK} S281C in detergent micelles had a single distance corresponding to an outward occluded conformation in DEER studies, as predicted by MMM, but spectral

simulations of the RT data indicated an inward open state was also present. This was only a minor species in a ratio of 1:0.29, but the only substrate bound structures of Glt_{TK} are outward facing. This reveals the presence of Na⁺-substrate bound Glt_{TK} in an inward facing conformation which has not previously been observed. However, a substrate-bound inward facing structure has been solved structurally for Glt_{Ph}. This could indicate the inward open conformation lies slightly higher in energy than the outward occluded and is therefore suppressed by cooling to 50 K for DEER measurements.

The cryo-EM structure for Na⁺-inhibitor bound Glt_{TK} had two of the three protomers with TBOA bound in an outward open conformation, with the third in an inward open conformation with Na⁺ bound. This asymmetry was observed in the distance distributions for Glt_{TK} S281C where results corresponded to outward occluded and inward open conformations. This was also observed in the spectral simulations with the τ_c values proposed to correspond to outward open and inward open in relative ratio of 1:0.7. The absence of TBOA for the third protomer in the cryo-EM structure may be a result of functional cooperativity which is a contested topic for the glutamate transporters.^{103,104,133} Negative cooperativity would indicate the binding of one DL-TBOA affects the affinity of binding the others, where only two DL-TBOA molecules have been shown to bind. Alternatively, it could be a result of steric hinderance due to the bulky benzyl groups of TBOA preventing all three protomers being in the outward facing conformation. In the case of steric hinderance, this could indicate the protein moves between inward and outward open states and cannot transition via intermediate, occluded conformations in a 'flip flop' type motion. It has been shown DL-TBOA prevents closure of the HP2 gate which is needed to access the intermediary conformations.

The asymmetry of protomer conformations observed in detergent micelles was replicated in proteoliposomes, SMALPs and nanodiscs for Glt_{TK} S281C. There were subtle differences in the spectral simulation results for some binding states, for example when Na⁺-TBOA bound in detergent micelles the dominant state was outward open, with less dominant conformation as inward open. In all of the

membrane-like environments, this was reversed and also reflected in the relative populations of the extracted distance distributions. Substrate bound Glt_{TK} S281C in SMALPs had multiple distance distributions and the spectral simulations also indicated an inward open conformation was dominant where outward occluded was observed in all other mimetics. This was also the highest populated state in the distance distributions (4 nm) contrasting to the outward occluded distance of 2.3 nm seen as the only distance for the other membrane mimetics. Na⁺-inhibitor bound Glt_{TK} S281C exhibited heterogeneity in all four membrane mimetics, further supporting the negative cooperativity or steric hinderance suggestions. For Na⁺-inhibitor bound Glt_{TK} S281C in liposomes and SMALPs the inward open conformation was dominant, with less dominant outward open (the reverse of detergent micelles) but data from nanodiscs indicated an occluded conformation was present. These differences confirm the presence of lipid bilayers and native lipids affect protein conformation during the transport cycle. Overall nanodisc spectral simulations and distance distributions had the highest similarity to detergent micelles.

In proteoliposomes, the lower protein concentration and shorter phase memory relaxation times meant that distance distributions terminated at shorter inter-spin distances compared to detergent micelles. The presence of the outward facing conformation in the distance distributions was much more subtle, with the largest spin population in the inward facing state. These results were confirmed in the EasySpin spectral simulations of the RT spectra. Other than the distances obtained for the Na⁺-TBOA bound state, all binding states closely aligned with detergent micelle results. The exception being the single distance observed for Na⁺-substrate bound Glt_{TK} S281C, with two conformations observed in the spectral simulation results.

Glt_{TK} S281C in SMALPs exhibited similar spectral line shapes for all binding states, resulting in similar EasySpin spectral fitting and suggested conformations. The distance distributions extracted from DEER spectroscopy were similar to detergent micelles, but the main difference observed was for substrate bound Glt_{TK} S281C in SMALPs where multiple distances were observed. A new peak around 3.5 nm was

observed, with the 2.4 nm (outward occluded) and 4 nm (inward open) distances still present. This could represent a previously unseen intermediate state as a result of the presence of native lipids, and potential conformational flexibility as a result. However, this was not observed in the cw-EPR spectral simulations and could be a transiently populated state with low functional occupancy at RT. These results are also not observed in nanodiscs which are a similar environment, with the difference being the native lipids in SMALPs. Alternatively, studies have suggested lateral pressure of the SMALP may limit conformational changes.¹⁸ This may account for the 4 nm distance observed in every sample as the main distance and also the lack of change in line shape at room temperature. The similar spectral line shapes for Glt_{Tk} S281C in SMALPs were also seen for Glt_{Tk} A367C in the same lipid environment. The distance distributions were almost identical for apo, Na⁺ bound and Na⁺-substrate bound states with only minor differences apparent in the Na⁺-inhibitor bound state suggesting the presence of multiple conformations. The transport and binding functionality of SMALPs needs to be tested (using transport assays and ITC) to validate these results for both Glt_{Tk} S281C and A367C samples.

The other five Glt_{Tk} variants proved to be less informative in both detergent micelles and proteoliposomes. Overall results still correlated with the presence of both inward and outward facing conformations for all variants based on EasySpin simulations of RT spectra. However, for some of the variants the distance distributions determined from the DEER traces did not reach the range predicted by MMM, and therefore could not be correlated to specific conformational changes.

Residue D298 in the Glt_{Tk} sequence is located between HP1 and TM7, in close proximity to substrate and ion binding sites. This residue was selected to inform on the intracellular side of the membrane during the transport cycle. *In silico* predictions based on cryo-EM and crystal structures revealed distance distributions >6 nm for inward facing conformations. The optimal phase memory time was too short to allow this to be observed. Apo Glt_{Tk} D298C revealed evidence of an outward facing conformation also seen when substrate and inhibitor bound, consistent with conclusions based on Glt_{Tk} S281C data. However, Na⁺-bound Glt_{Tk} D298C distance

distribution was cut off at 4.6 nm. It is predicted to adopt the inward facing conformations resulting in distances greater than the observable limit for these samples. The RT data is consistent with the presence of a second conformation with a distance too large to be observed by DEER. The cw-EPR spectra in proteoliposomes showed less residual MTSL compared to detergent micelles. The spectral line shape in the two membrane mimetics was similar, with less variation in τ_c and spectral line shape observed compared to Glt_{TK} S281C. DEER data for Glt_{TK} D298C in proteoliposomes was poor, with a very short Hahn echo phase memory time. This severely limited the time the Hahn echo could be observed for, and therefore the distance distributions that could be calculated were 4 nm maximum. The high noise levels in the raw data caused poor fitting during DeerNet interpretation and therefore high uncertainty in the distance distributions predicted. Na⁺-substrate and Na⁺-inhibitor bound Glt_{TK} D298C in proteoliposomes exhibited distances around 4 nm corresponding to an outward facing conformation, this could indicate the protein is in a more compact environment compared to detergent micelles which exhibited distances around 4.6 nm. The reconstitution protocol needs to be optimised for this variant and several others considering factors such as proteoliposome size (diameter), lipid composition and protein concentration.

Similarly, to Glt_{TK} D298C, both I312C and M314C proved difficult to extract meaningful distance distributions. I312C is located between two residues directly involved in Na⁺ binding. Residue M314C is part of the highly conserved NMDGT motif among the glutamate transporters, with residues directly involved in substrate and ion binding. These two residues are located on highly mobile parts of the protein, where residues are proposed to undergo rearrangement to optimise the position of substrate binding residues. RT cw-EPR spectra of I312C in both detergent micelles and proteoliposomes showed the presence of two different conformations in each binding state. Proteoliposome spectra differed from those in detergent micelles with slower τ_c s leading to the suggestion that either different conformational states were observed or the proteoliposome was a more compact environment causing the same conformational states to have slower τ_c s. Glt_{TK} M314C cw-EPR spectra showed changes in line shape between the different binding states, to a greater extent than

Glt_{TK} I312C and Glt_{TK} D298C. A mixture of species with different τ_c s were observed using EasySpin but assignment to specific conformations was challenging due to the small number of rotamers predicted in all MMM results for this variant. There were also high levels of residual MTSL, supporting the low number of rotamers and lowest protein yield of all the Glt_{TK} variants. The slowest τ_c was observed for the Na⁺-inhibitor bound state which could be a result of the bulky benzyl group of DL-TBOA in the substrate binding site in close proximity to this residue. Proteoliposomes showed slower τ_c s than detergent micelles but a similar change in values was observed, suggesting the same conformations were present but the spin label was in a more compact environment.

For both I312C and M314C the addition of d₈-glycerol had no effect on the T₂ determined by pulsed EPR methods. The typical oscillations in the presence of deuteration did not appear in the decay traces, indicating the spin-labelled residue was not accessible to the glycerol. This restricted the time the Hahn echo decay could be observed for in DEER experiments, again limiting the distances that could be determined. I312C showed shifts in the distance distribution during each stage of the transport cycle, indicating conformational change was occurring but the distance peaks were truncated due to the length of the time decay. On addition of Na⁺, the average observable distance increased and decreased again to the same value as the apo state on addition of substrate. Inhibitor bound Glt_{TK} I312C closely resembled Na⁺ bound. This pattern in distance changes was also observed for Glt_{TK} S281C. *In silico* analysis using MMM only predicted distance changes of 1 nm between the different conformations, which also overlapped, so assignment of the extracted distances was not possible. The same pattern was observed for I312C in proteoliposomes where an additional smaller distance was observed when substrate bound. This was the only sample for Glt_{TK} I312C where multiple distances were observed in a single sample. For M314C experimental data could not reach any of the distances predicted by *in silico* analysis (3.3–6.0 nm), and the extracted distance distributions also had high levels of uncertainty due to the noise in the DEER traces. So overall limited conclusions could be drawn but the data available were consistent with that for S281C and D298C. This was the case for both detergent micelle and proteoliposome

samples meaning distance distributions and conformational changes were not extracted.

Residue H334C in the Glt_{Tk} sequence was the most extracellular residue studied, located between TM7 and HP2. This residue varies between Glt_{Tk}, Glt_{Ph} and the EAAT's so it is not highly conserved. The cw-EPR spectra revealed similar spectral line shapes between detergent and proteoliposomes, with Na⁺ and Na⁺-inhibitor bound spectra being similar. This reflects the same two conformations present in the cryo-EM structure of Glt_{Tk} in these binding conditions. Apo and Na⁺-substrate also showed similarities. These similar pairings were observed for other variants too including Glt_{Tk} S281C in proteoliposomes. For the spectral simulations using EasySpin, each binding state had protein in two conformations. MMM predicted distances between 3.6–7.5 nm, however due to the relatively fast relaxation only distances up to 5 nm could be measured. In detergent micelles all species exhibited an intermediate state, with Na⁺ also including the inward facing state. Despite the cw-EPR spectra of Na⁺ and Na⁺-inhibitor bound Glt_{Tk} H334C having the same spectral line shape, this inward facing state was not observed when inhibitor bound. This could mean for the most extracellular residue, Glt_{Tk} H334C when bound to DL-TBOA shows a strong preference for outward facing conformations compared to the other variants. In proteoliposomes the low sample concentration and fast relaxation resulted in noisy DEER traces restricted to distances up to 4 nm. This precluded the possibility to observe inward facing states, meaning it remains unclear whether multiple conformations were present as predicted from simulations of RT data.

Glt_{Tk} A367C was the second most informative variant, after Glt_{Tk} S281C, and hence was studied in detergent micelles, proteoliposomes and SMALPs. This residue was part of HP2 which is proposed to be a gate to both intracellular and extracellular sides of the membrane. The distinct changes in cw-EPR spectral line shape and changing τ_c s correlated to different conformational states, including both outward and inward facing conformations. The results observed in proteoliposomes and detergent micelles were similar, with the *in silico* analysis predicted distance distributions between 2.6–5.0 nm, but generally broad distance distributions were observed which

could represent ensembles of similar conformations. Apo Glt_{Tk} A367C contained distances which corresponded to outward open, intermediate outward occluded and inward facing conformations; agreeing with the assignment from spectral simulations of RT cw-EPR. Addition of Na⁺ caused a shift to a slightly longer distance corresponding to that predicted for the inward open conformation. The intermediate outward occluded and outward open distances became more pronounced on addition of substrate, again corroborating assignments from EasySpin simulations of RT cw-EPR. The asymmetry observed for Glt_{Tk} S281C when Na⁺-inhibitor bound was replicated for Glt_{Tk} A367C in detergent micelles with the presence of an inward facing and outward facing state in both the distance distributions and simulation results, which also matched the cryo-EM structure. Due to the faster relaxation, proteoliposome samples could only be measured up to 4 nm. The same trend in distance changes was observed compared to detergent micelles but some distances were truncated due to the shorter distance limitation. However, results also indicate the lipid environment may cause a more compact protein structure, also reflected in slower τ_c s. However, Glt_{Tk} A367C in SMALPS exhibited similar spectral line shapes for all binding states, and little change in the distance distribution. These results should be validated using other techniques such as FRET, particularly given the results for Glt_{Tk} S281C in SMALPs closely resembled those of detergent micelles, proteoliposomes and nanodiscs. As with Glt_{Tk} S281C they also need to undergo functional testing such as binding and transport assays.

7.2 Investigating the binding interactions of Glt_{Tk} with L-glutamate

The archaeal homologues of the EAAT's have a higher binding affinity for L-aspartate than their human counterparts, with weak binding affinities observed for L-glutamate.¹¹¹ Glt_{Ph} has published K_D values for both L-aspartate and L-glutamate, however there is no evidence for the specific binding of L-glutamate at the substrate binding site of Glt_{Tk} despite the high sequence conservation between the two proteins at this region. A DEER experiment was recorded for Glt_{Tk} S281C with an excess of L-glutamate in the presence of Na⁺ ions. The distance distributions revealed a mixture of conformations with the highest population corresponding to an inward

open conformation and minor population as outward occluded. This contrasts to the single outward occluded conformation in the presence of L-aspartate. Addition of L-aspartate to the L-glutamate sample increased the proportion of outward occluded protein, but also broadened the distribution at higher distances corresponding to inward open conformation. This indicated there was not full displacement of L-glutamate by L-aspartate, but a binding interaction may be occurring. If no L-glutamate had bound, it would be expected that on addition of L-aspartate a single distance would be observed but the specific interaction between Glt_{Tk} and L-glutamate remains unclear. The non-specifically bound glutamate that isn't displaced by aspartate may prevent the protein from adopting its native distributions of conformations, accounting for the mixture of distances in the DEER experiment.

STD-NMR spectroscopy was applied to Glt_{Tk} to determine K_D values for both L-glutamate and L-aspartate. The EAAT's have been shown to transport L-glutamate and L-aspartate with similar affinities, contrasting to Glt_{ph} which has a higher affinity for the L-aspartate.^{84,86,133} However, the only reported substrates for Glt_{Tk} are D- and L-aspartate which were shown to have similar affinities and exhibited the same coupling stoichiometry of 3 Na⁺ per substrate molecule transported.¹¹³ Control experiments in the absence of Na⁺ confirmed the Na⁺-dependency of substrate binding with no saturation transfer observed between the protein and the ligand indicating no binding events. This is consistent with crystal structures where in the absence of Na⁺ the gate (HP2) is closed, occluding the substrate binding site from extracellular solution. The substrate binding site residues are not optimal for L-aspartate binding until local rearrangements are induced by binding of the first two equivalents of Na⁺.^{109,116}

The direct titration with L-glutamate showed as ligand concentration increased, the STD amplification factor increased. This means more ligand was binding to the protein and receiving saturation. Based on fractional saturation as a function of ligand concentration, it would be expected that the STD amplification factor would plateau in an excess of ligand due to the limited number of binding sites. So, at a given time, all binding sites would be occupied with ligand and the signal observed would reach

a maximum. However, this was not observed for Glt_{TK} with L-glutamate. Based on the STD curve obtained, a K_D of 11 mM was calculated (at 100 mM NaCl). This is in contrast to the same experiment on Glt_{ph} with a value of 414 μ M.¹³³ The lack of plateau in the STD curve (Figure 6.3) showed the substrate binding site did not reach a point of saturation in this experiment, and results indicated that non-specific binding could have occurred.

The non-specific binding proposal was supported by the results from the competition titration with L-aspartate - a proven high-affinity substrate of Glt_{TK}.^{109,113,116} The titration results found only 8% of L-glutamate was displaced by L-aspartate, with the remaining 92% L-glutamate still bound to the protein. This suggests in the direct L-glutamate titration only 8% of L-glutamate occupied the binding site, and the rest was interacting with the protein in other locations. When L-aspartate was added as a high affinity substrate, it displaced the L-glutamate to occupy the substrate binding site as shown by crystal structures. A K_D value of 17 nM for L-aspartate was calculated based on the competition titration which is lower than the range of published values for WT Glt_{TK}. However, this is calculated using the K_D value determined for the direct L-glutamate titration. The L-aspartate value is in the region of those calculated for Glt_{ph} using STD-NMR (42 nM and 13 nM for detergent micelles and liposomes respectively)¹³³ so this methodology could be repeated in liposomes where the presence of a lipid bilayer may prevent non-specific binding of glutamate.

K_D values for L-glutamate and L-aspartate with Glt_{TK} were determined using STD-NMR titrations. The binding interactions of L-glutamate are uncertain, meaning the validity of these results are questionable. Further research would be needed into the interactions of Glt_{TK} with L-glutamate to support or challenge the suggestion of non-specific binding, for example ITC and binding assays or smFRET as an alternative method of measuring distance changes. All STD NMR experiments presented should be repeated in liposomes, where previous studies of Glt_{ph} using STD-NMR found subtle differences compared to detergent micelles which indicated cooperative binding between the protomers which had not previously been observed.¹³³

7.3 Investigating the effect of Na⁺ concentration on protein conformation

Substrate binding for the glutamate transporters is strongly dependent on Na⁺ concentration.^{109,111,124,128} Isothermal titration calorimetry experiments of Glt_{Tk} showed that the binding affinity for Glt_{Tk} with L-aspartate is much weaker at sodium ion concentrations below 300 mM.¹¹⁶ This suggests for Glt_{Tk} a minimum sodium ion concentration of 300 mM may be needed for effective binding and transport. A comparison of the calculated distance distributions and cw-EPR spectra of Glt_{Tk} S281C was made using 50 mM Na⁺ and 300 mM Na⁺. Both the spectral line shapes and distance distributions were the same at both concentrations, highlighting that 50 mM Na⁺ was sufficient to investigate protein conformation and dynamics of Glt_{Tk}. The sodium ion concentration used in the STD NMR studies of both Glt_{Tk} and Glt_{ph} are low (100 mM) compared to other studies, but STD NMR is limited by the sodium ion concentration used due to difficulties with tuning at high salt concentrations.

7.4 Orthogonal labelling strategies for Glt_{ph} to achieve narrower distance distributions

SDSL of Glt_{ph} variants with MTSL followed by EPR measurements under various conditions (apo, Na⁺, Na⁺ with L-aspartate, and Na⁺ with DL-TBOA) provided evidence of structural rearrangements during the transport cycle. Subtle changes in line shape were observed using RT cw-EPR indicating conformational changes on binding ions and substrate/inhibitor molecules, as predicted using *in silico* analysis (MMM) in the form of the number of rotamers. DEER spectroscopy revealed conformational changes for MTSL labelled Glt_{ph} with a mixture of distance distributions observed in each binding state which are proposed to correspond to different conformations. This heterogeneity has been previously seen in other Glt_{ph} EPR experiments and structural studies.^{134,135} All extracted distance distributions for MTSL-labelled Glt_{ph} CM7 included a distance of 4.1 nm which is much higher than those predicted using MMM, this is thought to be an artefact of the experimental set up. Due to sample quality, only a short time to observe the Hahn echo was used; 4.1 nm is at the distance recognition limit and may not be real. Each of the four binding conditions contained

a distance around 2.7 nm thought to correspond to an outward facing conformation. Apo and Na⁺ bound distance distributions were very similar, indicating no large scale conformational rearrangements. Addition of both substrate and inhibitor results in a new distance at 1.8 nm which is thought to be an inward occluded conformation based on MMM predictions. The presence of both inward and outward facing confirmations confirmed the proposed alternating access mechanism where the rearrangement alternately exposes the substrate and ion binding sites to either side of the membrane.

UV-Visible spectroscopy and LT cw-EPR experiments confirmed the successful coordination of NTA to Cu²⁺ to form Cu²⁺-NTA. The results obtained are consistent with published studies used to optimize complex formation. LT cw-EPR and ESEEM was used to test the coordination of the complex to the dHis motif of Glt_{ph} CM7. The latter exhibited particularly clear results comparing Cu²⁺-NTA in buffer with Cu²⁺-NTA and Glt_{ph} CM7 (Figure 5.15). For Glt_{ph} CM7, nuclear modulations were present which had been previously shown to resemble imidazole coordination by Gamble et al.¹⁹¹ Future experiments could include applying ESSEM to quantify the number of ¹⁴N nuclei coupled to the Cu²⁺ centre, but for initial test experiments conducted here this was not needed.

DEER studies were used to detect conformational changes of Cu²⁺-NTA labelled Glt_{ph} CM7. However, the results exhibited low modulation depths, resulting in lower signal to noise, and short T₂ which limited the time the Hahn echo could be observed for and therefore the distance distributions that could be quantified using DeerAnalysis. None of the distances extracted from these experiments corresponded to those predicted by MMM based on crystal structures. Cu²⁺-NTA labelled Glt_{ph} CM7 distance determination experiments need to be optimized to increase the modulation depth and record higher quality data with lower noise levels, in addition to recording the Na⁺-inhibitor bound state. This would also work to corroborate the distance distributions extracted. Literature reports investigations of the effect of buffer on the binding efficiency and modulation depth in pulsed EPR spectroscopy where presence of free Cu²⁺-NTA reduced the modulation depth.¹⁹¹ The optimal buffer was found to

be MOPS, closely followed by phosphate and HEPES. TRIS was found to be degrading for Cu²⁺-NTA loading to the dHis site. For all EPR studies of Glt_{ph}, HEPES buffer was used. Optimisation of pH and buffer conditions was not attempted because the data quality of the preliminary measurements was of such a low standard that it is unlikely to produce such a significant improvement to the EPR results.

The final stage of the experiments was to orthogonally label Glt_{ph} CM7 using both MTSL (attaching to cysteine residues) and Cu²⁺-NTA (coordinate to dHis motif). The LT cw-EPR spectra appeared as a superposition of each of the spin-labelled samples respectively under the conditions used for Cu²⁺-NTA labelled protein. A DEER experiment was recorded for orthogonally labelled apo Glt_{ph} CM7 which revealed a wide array of distance distributions. This was predicted by MMM due to the presence of two spin labels per protomer. Deconvolution was difficult, and the wide distance distribution spread between 2.2–3.3 nm is proposed to include including inward occluded (2.5–2.8 nm), outward occluded (2.4 and 3.1 nm) and outward facing (3.4 nm) conformations.

Future experiments should include recording the distance distributions of orthogonally labelled Glt_{ph} CM7 in the different binding states to follow any binding-induced conformational changes. Results could be compared to previous studies to confirm the potential of the method even though the set up needs further optimisation. The wide spread of distance distributions may make deconvolution of the distances difficult, but this links back to the future application of inversion recovery-filtered iDEER to eliminate the observation of specific interactions and subsequent spectral filtering. This would simplify the overlap of spectral features in Cu²⁺-NTA – nitroxide interactions at low frequency. These initial experiments were also only tested on one of five possible Glt_{ph} variants. Glt_{ph} CM7 has the two labelling sites both located on the scaffold domain, meaning a lack of change in distance distributions and spin label environment was expected. The other variants have at least one of the two labelling sites located on a mobile part of the protein and therefore may show larger changes in spectral line shape and distance distribution

during the transport cycle. Therefore, once the methodology is optimised, all experiments should be repeated on the other Glt_{ph} variants.

The overall aim of this study was to introduce Cu²⁺-NTA as a spin-label for Glt_{ph} to yield narrower distance distributions than nitroxide based spin labels (MTSL). The ultimate aim is to use iDEER to selectively remove the interaction of pairs of spin-labels, deconvoluting the distance distributions (refer to Appendix A15). Further optimization of Cu²⁺-NTA is required before these studies are attempted, including investigation of other Glt_{ph} variants. Previous studies found that relaxation-induced dipolar modulation enhancement (RIDME) experiments had an increased sensitivity of a factor of 100 for Cu²⁺ labelled proteins compared to DEER experiments, particularly at Q-band frequencies.^{194,203} RIDME is an alternative pulsed EPR technique which replaces the pump pulse in DEER with a longitudinal relaxation block with no bandwidth limitations.²⁰³ This could be a point of investigation in the future.

7.5 Summary and future work

Overall the aims set out in Section 1.6 were achieved. This thesis describes the first application of EPR spectroscopy to elucidate the mechanism and associated conformational changes of Glt_{TK} during the transport cycle. Six Glt_{TK} variants were studied using a combination of cw- and pulsed EPR spectroscopy. Changes in the extracted rotational correlation times and inter-spin distances were correlated with the expected conformational changes upon binding substrate or inhibitor with co-transported Na⁺ ions. EPR results revealed the presence of both inward and outward facing conformations for the four binding conditions studied, including open and occluded states. These results corroborate the proposed alternating access mechanism based on other biophysical and structural studies (Figure 7.1). Current published structures are mostly outward facing conformations, with inward facing protomers only observed with Na⁺ and Na⁺-inhibitor bound. The presence of both inward and outward facing conformations in an apo state was confirmed using both RT cw-EPR spectral simulations and DEER distance distributions. There was a shift to majority inward facing protomers on the addition of Na⁺, which reverted back to

outward facing when substrate was added. Na⁺-inhibitor bound Glt_{Tk} included a mixture of both inward and outward facing states, as was observed in the cryo-EM structure, with further evidence pointing towards either steric hinderance or negative cooperativity preventing all protomers occupying an inhibitor bound outward facing conformation. These results support the current proposed alternating access mechanism, highlighting the transiently populated states of Glt_{Tk} which have not been observed using structural techniques. Each of the conformations in Figure 7.1 were observed, in at least one variant, with the addition of occluded conformations which are not reflected in the transport cycle shown. These occluded conformations are likely to occur when transitioning between open and closed conformations, reflecting the ability of EPR spectroscopy to capture short-lived states.

A comparison of different membrane mimetics revealed close correlation between nanodiscs and detergent micelles, whilst obtaining protein concentrations in proteoliposomes sufficient to generate reliable data proved difficult. Results using SMALP encapsulated Glt_{Tk} need to be validated using other techniques such as FRET, and assays to confirm substrate binding and transport. Therefore at present nanodiscs, which have been utilised to generate some of the published cryo-EM structures.¹²², appear to be the most suitable environment for EPR studies of EEATs and their homologues. Glt_{Tk} S281C in nanodiscs showed different line-shapes between the four binding conditions, reflected in the parameters extracted from spectral simulations. The data corresponded to the presence of three different protein conformations: inward facing, outward facing and outward occluded. Interestingly, when using DEER spectroscopy the substrate-bound inward facing conformation was only observed in the SMALP membrane mimetic for both Glt_{Tk} 281C and 367C highlighting the importance of the surrounding protein environment.

The STD-NMR experiments to calculate a K_D for L-aspartate and L-glutamate binding to Glt_{Tk} inconclusive. There are published K_D values for the binding of L-aspartate to Glt_{Tk} however due to the high affinity of this ligand the binding interaction could not be measured directly using STD-NMR. This required a competition titration using L-glutamate, for which the STD NMR titration was inconclusive. Despite having high

sequence identity in the substrate binding region to Glt_{Ph}, for which there are published K_D values with L-glutamate, the large apparent K_D values for L-glutamate with Glt_{Tk} suggest non-specific binding elsewhere on the protein precludes accurate determination of the binding affinity of this ligand to the substrate binding region of Glt_{Tk}. Investigating the changes in distance determination upon L-glutamate binding to Glt_{Tk} S281C also suggested a non-specific interaction was occurring.

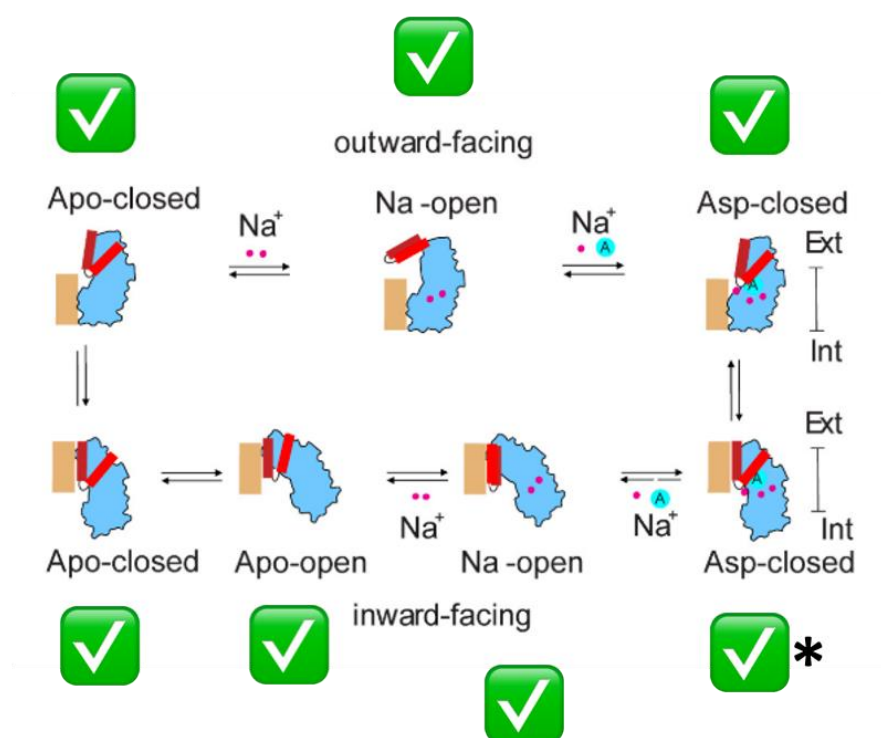


Figure 7.1 The proposed alternating access mechanism for the glutamate transporters and their identification using EPR spectroscopy. The scaffold domain is shown in beige which remains fixed during the transport cycle. The transport domain is shown in blue, with the gate (HP2) controlling access in red. Each state is labelled with the corresponding binding condition and protein conformation. Green ticks represent successful identification of the corresponding protein conformation using EPR spectroscopy in this thesis. Aspartate (Asp)-bound inward facing closed (*) conformation was only observed for Glt_{Tk} S281C in SMALPs using pulsed EPR spectroscopy.

Initial experiments on orthogonal labelling of Glt_{Ph} were conducted using both MTSL and Cu²⁺-NTA as spin labels. The goal was the use of a copper based spin label to extract narrower distance distributions using DEER. Pulsed EPR confirmed successful attachment of Cu²⁺-NTA to the dHis motif, but preliminary results of Cu²⁺-NTA labelled Glt_{Ph} CM7 showed the narrower distance distributions did not materialise

and the extracted distances did not correlate to those predicted using *in silico* analysis (MMM). Orthogonally labelled Glt_{ph} CM7 showed a large distribution of different distances, as expected with the presence of two spin labels per protomers. Overall, these results did not provide a strong foundation for further studies of Glt_{ph} spin labelled with Cu²⁺-NTA, at least for the variants studied in this thesis, and the specific techniques applied.

In this thesis, six variants were used which informed on different regions of the protein and therefore could only provide a generalised overview of conformational changes. Future work for this project could include designing new sets of variants at carefully selected locations to answer a more specific research question. For example, this could include residues near the proposed chloride channel to investigate the mechanism of chloride conductance. Positioning cysteine variants on the scaffold domain could also act as a control experiment to highlight the fixed position of these residues during the transport cycle. Designing a selection of variants to investigate a specific region of the protein (such as the HP2 gate) would allow a detailed insight into the local movements during the transport cycle. Although complex, it would also be interesting to study heteromeric Glt_{TK} variants which has previously been done for Glt_{ph}.¹⁴⁴ This was used to investigate cooperativity in substrate binding, which is a much debated topic in the glutamate transporters (as mentioned in Chapter 6).

Investigation into membrane mimetics was only briefly investigated in this thesis and could be an area of particular interest in the future. Within the scope of this project, the choice of detergent (such as different hydrophobic tail lengths) and lipids selected for solubilisation or reconstitution could be explored. This would alter the curvature of the micelle or liposome and therefore alter the surrounding local environment of the protein. SMALP-encapsulated Glt_{TK} was not tested for binding or transport in this thesis and would be a key experiment moving forwards to validate the EPR results presented. Research could also include looking into the number of surrounding lipids in the SMALP or the overall size of the discs. As research progresses, alternative mimetics to SMALPS which maintain the surrounding native lipids will be designed and be of particular interest in this area of research. Testing the binding and transport

would be essential for any new variants or membrane mimetics to confirm protein functionality, as well as checking protein folding and stability.

There is also scope for designing new EPR experiments for these Glt_{TK} variants. For example applying techniques to follow the conformational changes (on addition of sodium and/or substrate) between two specific conformational states. This could be via changes in spectral intensity or line shape, for example, using freeze-quenching in an attempt to trap the protein at small time increments as it changes conformation. This could reveal much smaller-scale transitions compared to those observed in DEER spectroscopy. Re-measurement of samples at higher frequency (pulsed Q-band or W-band) could also improve the quality of DEER traces. Although this is likely to provide significant improvements for variants such as Glt_{TK} M314C which generally had poor quality in all EPR experiments. To validate the distances measured using pulsed EPR spectroscopy, experiments could be replicated using FRET.

8 Materials and methods

8.1 Glt_{7k}

8.1.1 Plasmids

Plasmids pBAD24 containing the gene for C-terminally His₈-tagged Glt_{7k} were supplied by Albert Guskov and Dirk Slotboom (University of Groningen). This included wild type (WT), S281C, D298C, I312C, M314C, H334C and A367C.

8.1.2 Expression, purification, and site-directed spin labelling of Glt_{7k} in detergent

The expression vector was transformed into chemically competent MC1061 *Escherichia coli* cells using the heat-shock transformation (1 ng/μL, 100 μL).²⁰⁴

Cells containing the Glt_{7k} plasmid were grown in LB media (10 g peptone, 10 g NaCl, 5 g yeast extract per 1 L) containing ampicillin (100 μg/mL) at 37°C, 190 rpm until an OD₆₀₀ of 0.6–0.8 was reached. Gene expression from the pBAD24 plasmid was induced by the addition of L-(+)-arabinose (0.05%) and left for further incubation (180 minutes at 37°C, 190 rpm). Cells were harvested by centrifugation (Beckmann Avanti J-26) at 6000 rcf for 10 minutes and pellets were stored at -80°C.

Cells were resuspended in lysis buffer (20 mM Tris pH 8, 200 mM KCl, 5 mM MgCl₂, 0.4 mg/mL lysozyme, 0.02 mg/mL DNase, 10% glycerol, 1.05 mM phenylmethanesulfonyl fluoride, and 1 cOmpleteTM protease inhibitor cocktail tablet per 100mL buffer). The cells were lysed using LM20 microfluidizer[®] (Microfluidics, Westwood MA) at 18000 Psi for two cycles. The cell lysate and cell debris were separated using centrifugation (9000 rcf, 4°C for 30 minutes). Ultracentrifugation of the supernatant resulted in pelleted membranes (176000 rcf, 4°C for 2 hours) which were subsequently homogenised in buffer A (20 mM Tris pH 8, 200 mM KCl).

n-Dodecyl β -D-maltoside (DDM, Glycon Biochemicals, Germany) was added to a final concentration of 1% and protein was solubilised at 4°C for 60 minutes with gentle agitation. Meanwhile TALON Metal Affinity Resin was pre-equilibrated in buffer A (500 μ L resuspended resin per 1 L culture medium). Solubilised protein was separated from other cell debris by ultracentrifugation (176000 rcf, at 4°C for 20 minutes). Detergent concentration was diluted to 0.5% using buffer A supplemented with 10 mM imidazole (pH 8) before being added to pre-equilibrated TALON resin and left to bind (60 minutes at 4°C with gentle agitation). Wash 1 and wash 2 were performed using buffer A supplemented with 0.075% DDM and 10 mM imidazole (wash buffer 1, 40 column volumes) or 20mM imidazole (wash buffer 2, 5 column volumes) respectively. Protein was eluted in 10 x 1 mL fractions of elution buffer (buffer A supplemented with 0.075% DDM and 350 mM imidazole) and analysed by Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Detergent-solubilised protein was buffer exchanged into buffer B (10 mM HEPES pH 7.5, 100 mM NaCl (or KCl for apo conditions), 0.075% DDM) following standard manufacturer protocols using a Sephadex G-25 PD-10 desalting column (GE healthcare). Protein concentrations were determined by optical absorbance (280 nm) using a molar extinction coefficient for WT Glt_{TK} of 37360 M⁻¹ cm⁻¹ on a NanoDrop (ND-1000) spectrophotometer. Protein was concentrated using centrifugal Amicon Ultra-2 Centrifugal Filter Units (100 kDa cut off, regenerated cellulose membrane).

8.1.2.1 Site-directed spin labelling on-column

For site-directed spin labelling, the above protocol was modified. Following binding of protein to TALON resin, flow through was collected and resin washed with 20 column volumes of wash buffer 1. The protein-bound resin was left incubating overnight (in foil, at 4°C) with 2 column volumes of labelling buffer (buffer A supplemented with 0.075% DDM, 10 mM imidazole, 1 mM (1-Oxyl-2,2,5,5-tetramethyl- Δ 3-pyrroline-3-methyl) Methanethiosulfonate (MTSL). The resin was washed with 20 column volumes of wash buffer 1. Wash 2 and elution were conducted as described above.

8.1.2.2 Site-directed spin labelling after purification

For site-directed spin labelling of Glt_{Tk} after purification, MTSL was added in 10x excess per cysteine followed by overnight incubation (4°C, wrapped in foil, gentle agitation). Excess MTSL was removed using Zeba™ Spin desalting columns (Thermo Scientific) and buffer B. The post-labelling protein concentration was determined using a NanoDrop as previously described.

8.1.3 Functionality of spin labelled variants

Functionality assessments (isothermal titration calorimetry and uptake assay) were conducted by Emanuela Colucci at the University of Groningen

8.1.3.1 Isothermal titration calorimetry

Binding affinity of the Glt_{Tk} variants for L-Aspartate (L-Asp) was measured using isothermal titration calorimetry (ITC, TA instruments). In order to allow purified protein to bind to the substrate, 300 mM NaCl were added to the fraction and the protein was adjusted to the concentration of 8 µM using the same buffer for the dilution. The gel filtration buffer supplemented with 300 mM NaCl was also used to adjust the concentration of L-Asp to 100 µM. The protein was then added to the pre-equilibrated cell and kept at 25°C while stirring at 350 rpm. The substrate (at the initial stock concentration of 50 µM) was incrementally titrated into the cell in 25 injections of 2 µL each at intervals of 120 seconds. Data were fitted using the NanoAnalyze software.

8.1.3.2 Uptake assay

Proteoliposomes were prepared for the uptake assay by collaborators as per their protocol.¹¹⁹ An aliquot of 20 mg/mL *Escherichia coli* polar lipid extract in 50 mM potassium phosphate buffer (KPi) pH 7.0 was extruded 11 times through a 400 nm pore-size polycarbonate membrane. The lipid mixture was diluted to 4 mg/mL using

50 mM KPi pH 7.0 and destabilized with 10% Triton-X100 following absorption at 540 nm. The purified protein was then added to the solution in a ratio of 1:800 (w/w protein:lipid) and incubated for 30 minutes at room temperature on a rocking platform. Bio-Beads (Bio-Rad) were added to the lipid-protein mixture and incubated at 4°C: 25 mg/mL for 30 minutes, 15 mg/mL for 1 hour, 19 mg/mL overnight and 4 mg/mL for 2 hours. Bio-Beads were removed using filtration and the proteoliposomes were concentrated using ultracentrifugation (298,906 rcf, 4°C, 20 minutes). Pellets were resuspended to 20 mg/mL in internal lumen buffer (10 mM KPi pH 7.0, 300 mM KCl). Internal buffer was exchanged by three cycles of freeze-thawing. Aliquots were stored in liquid nitrogen until further use.

Proteoliposomes were thawed and collected by centrifugation (298,906 rcf, 4°C, 20 minutes). Following resuspension in internal lumen buffer, the proteoliposomes were extruded as previously described. Centrifugation was repeated and pellets were resuspended in the same internal lumen buffer to 100 mg/mL. To record each point of the sodium dependence experiment, 2 µL of proteoliposomes were diluted 100 times in the reaction buffer 10 mM KPi, pH 7.0, X mM NaCl, (300 mM – X) mM Choline Chloride, 500 nM valinomycin, 500 nM L-aspartate, 500 nM ¹⁴[C]-L-aspartate. After 30 seconds from the start of the experiment, the 200 µL reaction was quenched in 2 mL of ice-cold buffer (10 mM KPi pH 7.0, 300 mM KCl) and filtered through a nitrocellulose filter (Protran BA 85- Whatman filter). These steps were repeated for all the sodium concentrations (0, 1, 10, 30, 75, 250 mM) and filters were dissolved in a scintillation cocktail. Radioactivity was then measured by counting each point for 2 minutes using a PerkinElmer Tri-Carb 2800RT scintillation counter.

8.1.4 Detergent-free environments

8.1.4.1 SMALP encapsulation

Styrene-maleic acid (SMA) 30010P (2.3:1 styrene:maleic acid, Polyscope, Netherlands) was added to a final concentration of 1.5% (v/v) and protein was solubilised at room temperature for 2 hours with gentle agitation. Meanwhile Ni

Sepharose 6 Fast Flow resin was pre-equilibrated in buffer A (500 μ L resuspended resin per 1 L culture medium). Solubilised protein was separated from other cell debris by ultracentrifugation (220000 rcf, at 4°C for 20 minutes). Supernatant was concentrated using Amicon Ultra-15 Centrifugal Filter Units (100 kDa cut off, regenerated cellulose) and diluted by adding an equal volume of buffer A. SMA lipid nanoparticle (SMALP) encapsulated Glt_{Tk} was bound to the pre-equilibrated Ni Sepharose resin overnight at 4°C, with gentle agitation. Wash 1 and wash 2 were performed using buffer A supplemented with 10 mM imidazole (wash buffer 1, 60 column volumes) and 20mM imidazole (wash buffer 2, 8 column volumes) respectively. Protein was eluted in 10 x 1mL fractions of elution buffer (buffer A supplemented with 350 mM imidazole) and analysed by SDS-PAGE. SMALP Glt_{Tk} samples were buffer exchanged into buffer C (10 mM HEPES pH 7.5, 100 mM KCl) following standard protocols using a Sephadex G-25 PD-10 desalting column (GE healthcare) and concentrated as previously described for detergent solubilised Glt_{Tk}.

8.1.4.2 Preparing proteoliposomes

For reconstitution into liposomes, protein was first concentrated to approximately 50 μ M using Amicon Ultra-2 Centrifugal Filter Units. Preparation of lipids was performed under inert gas (argon) at all stages. A mixture (3:1 ratio) of *Escherichia coli* polar lipid extract and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) lipids was dried under argon for 1 hour, forming a thin lipid film. Degassed buffer (20mM HEPES pH 7.8, 100mM NaCl) was added to give a final lipid concentration of 20 mg/mL. The resuspended lipids underwent 3 freeze-thaw cycle to form large multilamellar vesicles, which were then extruded (11 times, Avanti mini-extruder) using a 400 nm pore-size polycarbonate membranes to form large unilamellar vesicles. The liposomes were diluted to 4 mg/mL using buffer supplemented with 25% glycerol. Liposomes were destabilised through addition of degassed Triton X-100 (10% w/v, 150 μ L per 5 mL of liposomes). Concentrated protein (25 μ g per mg lipid) was added to the detergent-destabilised liposomes and left to incubate (30 minutes, 4°C). Four consecutive additions of pre-equilibrated Bio-Beads SM-2 (200 mg per 5mL mixture) were added to the protein-liposome suspension (1 addition per hour, then

a final overnight addition) before being removed by filtration. The proteoliposome suspension was concentrated using ultracentrifugation (160000 rcf, 4°C, 2 hours) and the resultant proteoliposome pellet resuspended to a final protein concentration ~80 µM in detergent-free D₂O buffer: either 20 mM HEPES pH 7.8, 100 mM KCl or 20 mM HEPES pH 7.8, 100 mM NaCl. For substrate- and inhibitor-bound samples these resuspended lipids were saturated with 1 mM L-aspartate or 1 mM DL-*threo*-β-Benzyloxyaspartic acid (DL-TBOA) respectively (1 hour). Proteoliposome samples were loaded into EPR tubes and stored in liquid nitrogen.

The reconstitution density ($\chi_{reconstituted}$) of the proteoliposomes can be calculated using Equation 8.1 where the [P] and [L] represent the concentration of protein and lipid respectively, and M_r^P and M_r^L the respective molecular weights. The molecular weight of the lipid mixture can be calculated from the combination of the *Escherichia coli* polar extract phospholipid profile (67% w/w) and POPC (33%, 760 g/mol). The relative composition of *Escherichia coli* polar lipid extract is phosphatidylethanolamine (PE, 67%), phosphatidylglycerol (PG, 23.2%) and cardiolipin (CA, 9.8%) with a molecular weight of 786 g/mol – which is the same as the lipid DOPC.²⁰⁵ This gives the overall average molecular weight of the lipid mixture as 777.42 g/mol. During reconstitution, 25 µg of protein was added per mg lipid. Given the molecular weight of Glt_{Tk} is 44642 g/mol, a reconstitution density of 1.39 x10⁻⁴ subunits/lipid.

$$\chi_{reconstituted} = \frac{[P] / M_r^P}{[L] / M_r^L}$$

8.1

The number of protein molecules per liposome can also be calculated. First the number of lipids in a liposome of known diameter must be determined (Equation 8.2). Liposomes were extruded using a 400 nm pore size membrane and therefore this was used as the diameter; h is the thickness of the bilayer which is typically around 5 nm; and a is the lipid head area group (0.7 nm for POPC and DOPC).²⁰⁶ This

gives the number of lipid molecules per 400 nm liposome (N_{tot}) as 1400701. When multiplied by $\chi_{reconstituted}$, it gives 196 as the number of protein molecules per liposome.

$$N_{tot} = \frac{\left[4\pi\left(d/2\right)^2 + 4\pi\left(d/2 - h\right)^2\right]}{a}$$

8.2

8.1.4.3 Reconstitution into nanodiscs

This was completed by Emanuela Colucci and Egor Marin at the University of Groningen

Proteoliposomes were prepared for the uptake assay by collaborators as per their protocol. An aliquot of 20 mg/mL *Escherichia coli* polar lipid extract in 50 mM potassium phosphate buffer (KPi) pH 7.0 was extruded 11 times through a 400 nm pore-size polycarbonate membrane. The liposomes were destabilised with 30 mM DDM, vortexed for 1 minute and incubated with gentle agitation (3 hours, 4°C). Purified Glt_{TK} variants were mixed with the destabilised lipids in a 3:5 molar ratio (protein:lipids) and left on ice for 30 minutes. Belt protein membrane scaffold protein 2N2 (MSP2N2), previously purified in buffer D (20 mM Tris, 100 mM KCl pH 8) and cleaved from the His-tag, was added to the mixture in a final molar ratio of 3:5:100 (Glt_{TK}:lipids:MSP2N2). The mixture was left to incubate at 4°C for 90 minutes. Detergent was removed by addition of Bio-Beads (700 mg/mL) and left incubate overnight at 4°C. Nanodiscs were formed upon removal of the detergent. Bio-Beads were removed, and the mixture was bound to pre-equilibrated Ni⁺ Sepharose resin (500 µL in buffer D) for 1 hour. A wash step was performed using buffer D supplemented with 30 mM imidazole. Protein was eluted in buffer D supplemented with 500 mM imidazole. The eluted nanodiscs were then further purified from imidazole by size exclusion chromatography on the Superdex 200 10/300 gel-filtration column (GE Healthcare) in 10 mM HEPES-KOH pH 8.0, 100 mM KCl. Nanodiscs were concentrated as previously described for detergent samples.

8.2 Glt_{ph}

8.2.1 Plasmids

C-terminally His₈-tagged Glt_{ph} inserted into the pBAD24 plasmid was supplied by Christopher Mulligan (University of Kent). This included C321S G280C F50H L54H (CM3), C321S G149C, V51H, K55H (CM7), C321S T375C V51H K55H (CM8), C321S G351C K132H D136H (CM10) and C321S R52C A364H E368H (CM11). Wild-type (WT) Glt_{ph} in pBAD24 plasmid was supplied by Tomas Stockner (Medical University of Vienna).

8.2.2 Expression, purification, and site-directed spin labelling of Glt_{ph} in detergent

The expression vector was transformed into chemically competent TOP10 *Escherichia coli* cells using the heat-shock transformation (1 ng/μL, 100 μL).

Protocol for cell growth follows that of Glt_{tk} with slight modification. Induction used 0.2% L-(+)-arabinose followed by a reduction in temperature to 30°C for 3 hours before cell harvesting. Cell lysis and detergent purification was amended with substitution of 20 mM HEPES pH 7.5 in lysis buffer and buffer A (instead of 20 mM Tris pH 8). For site-directed spin labelling the same protocol applies as for Glt_{tk}. For MTSL-labelled Glt_{ph} labelling was completed as part of the purification process, as detailed for Glt_{tk}.

Detergent-solubilised protein was buffer exchanged into 20 mM HEPES pH 7.5, 100 mM KCl (or NaCl), 0.075% DDM) following standard protocols using a Sephadex G-25 PD-10 desalting column (GE healthcare). Protein concentrations were determined by optical absorbance (280 nm) using a molar extinction coefficient for WT Glt_{ph} of 26820 M⁻¹ cm⁻¹ on a NanoDrop (ND-1000 spectrophotometer). Protein was concentrated using centrifugal Amicon Ultra-2 Centrifugal Filter Units (100 kDa cut off, regenerated cellulose membrane).

8.2.3 His-tag cleavage

Purified protein was incubated with bovine thrombin (10 U thrombin per mg protein) and 5 mM calcium chloride at room temperature, overnight with gentle agitation. SDS-PAGE was used to check successful his-tag cleavage reaction, followed by addition of 10 mM Ethylenediaminetetraacetic acid (EDTA) and 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF) to stop thrombin digestion. The reaction was passed through Zeba™ Spin desalting columns, concentrated as previously described and passed through pre-equilibrated TALON Metal Affinity Resin to ensure removal of any cleaved his-tags and un-cleaved protein. Protein concentration was determined using a NanoDrop using the parameters detailed in the previous section.

8.3 Preparation and analysis of Copper (II) NTA

8.3.1 Formation of copper (II) NTA

Copper (II) chloride stock (CuCl_2 , 100 mM) and copper (II) sulphate stocks (CuSO_4 , 100 mM) were prepared in ultrapure water and adjusted to pH 2. Nitrilotriacetic acid (NTA, 100 mM) was prepared in ultrapure water and adjusted to pH 12.

The NTA stock was diluted 1:8 in Glt_{ph} buffer A before adding 1 part CuCl_2 dropwise, giving a 10 mM Cu^{2+} -NTA stock which was used for addition to protein samples.

Samples for UV-Vis spectroscopy and EPR studies included: (i) CuCl_2 (10 mM in Glt_{ph} buffer) and (ii) Cu^{2+} -NTA (10 mM, prepared as described above)

8.3.2 UV-Visible spectroscopy of copper (II) NTA

Formation and behaviour of the Cu^{2+} -NTA stock was investigated using UV-Vis spectroscopy (Perkin Elmer Precisely Lambda 35). Spectra were recorded in QS high precision cells (1 mm, quartz, Hellma analytics) between 900–200 nm.

8.3.3 Spin labelling Glt_{ph} with copper (II) NTA

1 equivalent of Cu²⁺-NTA per double histidine (dHis) motif was added to purified Glt_{ph}. Samples were then incubated at 4°C for 30 minutes.

8.4 EPR Spectroscopy

8.4.1 Data acquisition

8.4.1.1 Sample preparation

For apo/Na⁺/Na⁺+substrate/Na⁺+inhibitor bound samples, spin-labelled spin labelled (MTSL bound) protein was used at concentrations ~80 µM. For the Na⁺-bound states, samples were supplemented with NaCl (50 mM) and incubated on ice for 1 hour. Substrate and inhibitor bound samples were prepared by addition of NaCl (50 mM) and either L-aspartate (1 mM) or DL-TBOA (1 mM).

For room temperature continuous (cw-) EPR experiments both detergent solubilised and proteoliposome samples (10 µL) were loaded into capillary tubes and inserted into sample tubes, sealed with parafilm. For low-temperature X-band EPR and DEER experiments both detergent solubilised and proteoliposome samples (~70 µL) were loaded in quartz EPR tubes (O.D. 4 mm, 250 mm length, Wilmad) and flash frozen in liquid nitrogen. Detergent solubilised samples had subsequent addition of 30% d₈-glycerol. For low-temperature Q-band EPR, samples (30 µL) were loaded into quartz Suprasil EPR tubes (O.D. 3 mm, 159 mm length, Wilmad).

8.4.1.2 Room temperature X-band cw-EPR measurements

Room temperature continuous-wave (cw-) EPR spectra at 9 GHz (X-band) were recorded using a Bruker Elexsys E500 spectrometer equipped with a Bruker ER4123D dielectric resonator. The microwave frequency was recorded using an external

frequency counter (macroni instruments 20 GHz microwave counter 2440ake model). Experimental parameters: microwave power, 0.2 mW; field modulation amplitude, 0.2 mT; field modulation frequency, 100 kHz; at ambient temperature. The number of scans recorded varied depending on sample concentration. The incident microwave frequency recorded during measurements was ~9.759 GHz. Spin labelling efficiencies were calculated by comparing the double integral of the respective cw-EPR spectra (first derivative) with known concentrations of TEMPO standards (10 μ M, 25 μ M, 50 μ M, 75 μ M, 100 μ M). The labelling efficiency was determined using protein concentration and the calibration curve constructed from TEMPO samples.

8.4.1.3 Low temperature X-band cw-EPR measurements

Low temperature cw-EPR spectra at X-band were recorded using a Bruker Elexsys E500 spectrometer equipped with a Bruker super-high Q (SHQ 4112SHQE/0144) resonator mounted on an Oxford ESR900 helium flow cryostat. A liquid-helium transfer set up was used with a Leybold PT-50 dry divac pump and proportional-integral-drive (PID) temperature controller. Helium flow was controlled by a needle valve on the transfer line and gas flow controller. The low temperature control system in the resonator involves a quartz dewar and sample holder which maintains a temperature controlled environment for the sample using continuous flow of helium gas. The microwave frequency was recorded using an external frequency counter (macroni instruments 20 GHz microwave counter 2440ake model). Experimental parameters for spin-labelled samples: microwave power, 0.0125 mW; field modulation amplitude, 0.2 mT; field modulation frequency, 100 kHz; centre field, 3364 G; sweep width, 180 G; temperature, 50K . The number of scans recorded varied depending on sample concentration. Experimental parameters for copper samples: microwave power, 0.9985 mW; field modulation amplitude, 0.4 mT; field modulation frequency, 100 kHz; centre field, 3050 G; sweep width, 1500 G; temperature, 10 K. The number of scans recorded varied depending on sample concentration.

8.4.1.4 Low temperature Q-band cw-EPR measurements

Low temperature cw-EPR spectra at Q-band were recorded using the same liquid-helium transfer set up as low temperature X-band above. A Bruker Elexsys E560 spectrometer equipped with a Bruker 5106 QTW resonator was mounted on an Oxford ESR900 helium flow cryostat. The microwave frequency was recorded using an external frequency counter (macroni instruments 20 GHz microwave counter 2440ake model); around 34 GHz for the samples recorded. Experimental parameters for spin-labelled samples: microwave power, 0.0006145 mW; field modulation amplitude, 0.2 mT; field modulation frequency, 100 kHz; centre field, 12127 G; sweep width, 150 G; temperature, 50 K. The number of scans recorded varied depending on sample concentration.

8.4.1.5 Pulsed X-band EPR spectroscopy

All pulsed EPR (DEER) experiments were performed at X-band on a Bruker Elexsys 680 spectrometer equipped with a Bruker ER4118 flexline resonator and a home-built closed-cycle refrigeration displacer unit.²⁰⁷ All microwave pulses were amplified by a 1 kW TWT amplifier (Applied Systems Engineering). Spin-labelled samples were measured at 50 K and copper-containing samples at 20 K.

Initial field-swept echo detected EPR and T_2 relaxation experiments were recorded using a standard Hahn-echo two pulse sequence: $\pi/2(10\text{ ns}) - \tau(120\text{ ns}) - \pi(20\text{ ns}) - \tau -$. These experiments were used to optimise DEER parameters. Experiments were conducted with shot repetition time of 2 ms and 50 shots per point. The field swept echo (FSE) was recorded as a function of magnetic field over 400 points, with a 200 G sweep width and 3050 G centre field for spin-labelled samples (3050 G centre field, 1500 G sweep width for copper samples), 48 dB video gain and 4–6 dB attenuation. These parameters were optimised for each sample. The FSE experiment was used to select the magnetic field with the maximum echo intensity for the Hahn echo phase memory time experiment (which will be referred to as the T_2 experiment/measurements), and to select the pump and detect positions for the

DEER experiment. The T_2 measurements were conducted as a function of time, over 700 points and the same settings as above. The π -pulse was displaced incrementally through a set number of points (every 8 ns) leading to a decrease in echo intensity over time.

The conventional 4-pulse dead-time free DEER sequence was used.²⁰⁸ Microwave mw_{n1} observer pulses ($\pi/2$ and π -pulses) were optimised to 20 ns in length, and a pump π -pulse of 16 ns and τ_1 of 120 ns used. The mw_{n2} pump π -pulse was stepped in 12 ns increments (i.e. t) through a τ_2 that varied depending on sample/measurement (typically between 0.75–2.5 μ s). Typically, the mw_{n1} observer frequency was matched to the maximum of the MTSL spectrum, and the mw_{n2} pump frequency set to the low-field edge of the MTSL spectrum (i.e. +70 MHz for spin-labelled samples, and +150 MHz for copper samples). Appropriate phase-cycling and tau-suppression protocols were used to collect the data using the Bruker PulseSPEL package. Experiments were conducted with shot repetition time of 2 ms and 50 shots per point.

8.4.2 Data analysis

EasySpin

EPR data analysis was performed using the MATLAB[®]-based package EasySpin for continuous-wave spectra.²⁰⁹ Fitting scripts were written in MATLAB using the EasySpin `chili` function for room temperature EPR spectra, and `pepper` for low temperature EPR spectra with the experimental parameters included. Two or three species were incorporated to account for free-MTSL present in the spin-labelled samples, and the relative weighting varied to determine the amount of free-label present. Low temperature spectra were first simulated to determine A_{zz} values which were then used to limit their variation in the simulations of room temperature spectra.

- Different systems or species were defined as 'SysX', where X represents any one system.

- For each system or species, start values were assigned and given a range to allow the parameter to change to find the optimum fit. This was determined by the 'Vary' commands.
- 'Exp' prefix includes experimental parameters such as microwave frequency, centre field, sweep width and the number of points.
- FitOpt is the chosen fitting algorithm.

Once 'best fit' values were obtained, the values were manually changed in the script before next fitting iteration. Once final fitting complete saved output for plotting.

DeerAnalysis

Pulsed EPR data analysis was performed using the MATLAB[®]-based package DeerAnalysis2019.¹⁶⁴ To obtain distance distributions a neural network approach, DeerNet, was used which gives 95% confidence bounds represented by grey areas in the relevant figures.¹⁶⁸ This method loads in the recorded pulsed EPR data and calculates relative distance distributions using algorithms within the software.

MMM

Predicted distance distributions and rotamer libraries were calculated using multiscale molecular modelling (MMM) software within MATLAB. Protein data bank (PDB) access codes were used as model systems to load in the protein structure. Specific residues were computationally mutated to a cysteine and subsequently spin labelled through *in silico* attachment of MTSL. The software produces an output file containing the number of positions MTSL can sample (rotamers) at a given site, as well as the predicted distance distributions between the spin-labelled sites. The spin-labelled structure can also be saved for use in visualization software of the MTSL rotamers.¹⁸³

8.5 NMR spectroscopy

8.5.1 Sample preparation

WT Glt_{TK} and Glt_{Ph} were produced and purified as previously described (8.1.2 and 8.2.2 respectively), with the following buffer modifications: 20 mM KPi pH 7.5 instead of HEPES or Tris and 100 mM KCl. Purified protein was exchanged into 20 mM KPi pH 7.5, 100 mM NaCl (or KCl for control experiments), 0.075% DDM in D₂O. Protein samples were concentrated to 20 μ M and 0.5 mL was loaded into tubes: Wilmad® NMR precision tubes.

8.5.2 Data acquisition

Saturation transfer difference nuclear magnetic resonance (STD NMR) spectroscopy was carried out under the conditions optimised and published previously in the laboratory.¹³³ All experiments were conducted at 278 K in D₂O buffer on an ultra-compact 800 MHz Bruker Avance III NMR spectrometer, equipped with an inverse tripled resonance HR-MAS probe. All STD experiments were set up with an on-resonance frequency at 0.6 ppm, to selectively saturate aliphatic side chains of the protein, and an off-resonance frequency at 40 ppm. For selective saturation a 49 ms long gaussian shaped pulse was used with a 1 ms delay between successive pulses. Excess magnetisation was removed using two trim pulses (2.5 and 5 ms) followed by a 3 ms gradient pulse on the z-axis. The saturation time was kept constant at 2 s with recycling delay of 2 s. STD experiments consisted of 512 scans with a varying number of loops depending on ligand concentration to achieve clearly resolved NMR peaks. The difference between the resultant 2D spectra for on- and off-resonance experiments yielded the STD spectrum for each dataset and titration point. Three control experiments were conducted to rule out non-specific interactions: (A) Buffer + 1 mM L-glutamate (B) Buffer + 1 mM L-aspartate and (C) WT Glt_{TK} in KCl buffer + 0.5 mM L-glutamate. K_D values for the titrations detailed below were calculated using the curve fitting toolbox within MATLAB.

For K_D determination of WT Glt_{7k}, L-glutamate was added in concentrations ranging between 0 to 10 mM via titration. A competition-based titration with L-aspartate, ranging from 0 to 1 μ M, with WT Glt_{7k} supplemented with 5 mM L-glutamate was conducted. In addition, a direct titration of L-aspartate with WT Glt_{7k} under the same conditions previously described for L-glutamate.

WT Glt_{ph} and Glt_{ph} C321A A71C underwent a direct titration with L-glutamate consisting of additions ranging between 0 to 5 mM to investigate the difference between WT and a variant. Buffers were modified to contain different sodium ion concentrations (50 mM, 100 mM, and 150 mM) to see the effect on K_D of WT Glt_{ph} and L-glutamate – using ligand concentrations between 0 to 5 mM.

9 Bibliography

1. Hedin, L. E., Illergård, K. & Elofsson, A. An introduction to Membrane Proteins. *J. Proteome Res.* **10**, 3324 (2011).
2. Bosshart, P. & Fotiadis, D. Secondary active transporters. in *Bacterial Cell Walls and Membranes* (Springer, 2019).
3. Hu, C., Tao, L., Cao, X. & Chen, L. The solute carrier transporters and the brain: Physiological and pharmacological implications. *Asian J. Pharm.* **15**, 131 (2020).
4. Saier, M. H. *et al.* The transporter classification database (TCDB): 2021 update. *Nucleic Acids Res.* **49**, D461 (2021).
5. Hediger, M. A., Clémenton, B., Burrier, R. E. & Bruford, E. A. The ABCs of membrane transporters in health and disease (SLC series): Introduction. *Mol. Asp. Med.* **34**, 95 (2013).
6. Fichant, G., Basse, M. J. & Quentin, Y. ABCdb: An online resource for ABC transporter repertoires from sequenced archaeal and bacterial genomes. *FEMS Microbiol. Lett.* **256**, 333 (2006).
7. Ren, Q., Chen, K. & Paulsen, I. T. TransportDB: A comprehensive database resource for cytoplasmic membrane transport systems and outer membrane channels. *Nucleic Acids Res.* **35**, 274–279 (2007).
8. Krogh, A., Larsson, B., Von Heijne, G. & Sonnhammer, E. L. L. Predicting transmembrane protein topology with a hidden Markov model: Application to complete genomes. *J. Mol. Biol.* **305**, 567 (2001).
9. Wallin, E. & Von Heijne, G. Genome-wide analysis of integral membrane proteins from eubacterial, archaean, and eukaryotic organisms. *Protein Sci.* **7**, 1029 (1998).
10. Puthenveetil, R., Christenson, E. T. & Vinogradova, O. New Horizons in Structural Biology of Membrane Proteins: Experimental Evaluation of the Role of Conformational Dynamics and Intrinsic Flexibility. *Membranes.* **12**, 227 (2022).
11. Overington, J. P., Al-Lazikani, B. & Hopkins, A. L. How many drug targets are there? *Nat. Rev. Drug Discov.* **5**, 10 (2006).

12. Hofmann, L. & Ruthstein, S. EPR Spectroscopy Provides New Insights into Complex Biological Reaction Mechanisms. *J. Phys. Chem. B* **126**, 7486 (2022).
13. Skrzypek, R., Iqbal, S. & Callaghan, R. Methods of reconstitution to investigate membrane protein function. *Methods* **147**, 126 (2018).
14. Seddon, A. M., Curnow, P. & Booth, P. J. Membrane proteins, lipids and detergents: Not just a soap opera. *Biochim Biophys Acta Biomemb* **1666**, 105 (2004).
15. Iwahashi, Y. & Nakamura, T. Orientation and reactivity of NADH kinase in proteoliposomes. *J. Biochem.* **105**, 922 (1989).
16. Yeh, V., Goode, A. & Bonev, B. B. Membrane protein structure determination and characterisation by solution and solid-state NMR. *Biology*. **9**, 396 (2020).
17. Bayburt, T. H., Grinkova, Y. V. & Sligar, S. G. Self-Assembly of Discoidal Phospholipid Bilayer Nanoparticles with Membrane Scaffold Proteins. *Nano Lett.* **2**, 853 (2002).
18. Pollock, N. L., Lee, S. C., Patel, J. H., Gulamhussein, A. A. & Rothnie, A. J. Structure and function of membrane proteins encapsulated in a polymer-bound lipid bilayer. *Biochim Biophys Acta Biomemb* **1860**, 809 (2018).
19. Lee, S. C. *et al.* A method for detergent-free isolation of membrane proteins in their local lipid environment. *Nat. Protoc.* **11**, 1149 (2016).
20. Knowles, T. J. *et al.* Membrane proteins solubilized intact in lipid containing nanoparticles bounded by styrene maleic acid copolymer. *J. Am. Chem. Soc.* **131**, 7484 (2009).
21. Jamshad, M. *et al.* G-protein coupled receptor solubilization and purification for biophysical analysis and functional studies, in the total absence of detergent. *Biosci. Rep.* **35**, e00188 (2015).
22. Oluwole, A. O. *et al.* Solubilization of Membrane Proteins into Functional Lipid-Bilayer Nanodiscs Using a Diisobutylene/Maleic Acid Copolymer. *Angew. Chem. Int. Ed.* **56**, 1919 (2017).
23. Kermani, A. A. A guide to membrane protein X-ray crystallography. *FEBS J.* **288**, 5788 (2021).
24. Cheng, Y. Membrane protein structural biology in the era of single particle cryo-EM. *Curr. Opin. Struct. Biol.* **52**, 58 (2018).

25. Callaway, E. Revolutionary cryo-EM is taking over structural biology. *Nature* **578**, 201 (2020).
26. Puthenveetil, R. & Vinogradova, O. Solution NMR: A powerful tool for structural and functional studies of membrane proteins in reconstituted environments. *J. Biol. Chem.* **294**, 15914 (2019).
27. Bartels, K., Lasitza-Male, T., Hofmann, H. & Löw, C. Single-Molecule FRET of Membrane Transport Proteins. *ChemBioChem* **22**, 2657 (2021).
28. Gilbert, B. C., Murphy, D. M. & Chechik, V. *Electron Paramagnetic Resonance*. (The Royal Society of Chemistry, 2015).
29. Claxton, D. P., Kazmier, K., Mishra, S. & McHaourab, H. S. Navigating Membrane Protein Structure, Dynamics, and Energy Landscapes Using Spin Labeling and EPR Spectroscopy. *Methods Enzym.* **564**, 349 (2015).
30. Thaker, T. M. *et al.* Asymmetric drug binding in an ATP-loaded inward-facing state of an ABC transporter. *Nat. Chem. Biol.* **18**, 226 (2022).
31. Wegener, C., Tebbe, S., Steinhoff, H. J. & Jung, H. Spin labeling analysis of structure and dynamics of the Na⁺/proline transporter of *Escherichia coli*. *Biochemistry* **39**, 4831 (2000).
32. Jeschke, G., Wegener, C., Nietschke, M., Jung, H. & Steinhoff, H. J. Interresidual Distance Determination by Four-Pulse Double Electron-Electron Resonance in an Integral Membrane Protein: The Na⁺/Proline Transporter PutP of *Escherichia coli*. *Biophys. J.* **86**, 2551 (2004).
33. Hilger, D., Polyhach, Y., Jung, H. & Jeschke, G. Backbone structure of transmembrane domain IX of the Na⁺ /proline transporter PutP of *Escherichia coli*. *Biophys. J.* **96**, 217 (2009).
34. Olkhova, E., Raba, M., Bracher, S., Hilger, D. & Jung, H. Homology model of the Na⁺/proline transporter PutP of *Escherichia coli* and its functional implications. *J. Mol. Biol.* **406**, 59 (2011).
35. Claxton, D. P. *et al.* Ion/substrate-dependent conformational dynamics of a bacterial homolog of neurotransmitter:sodium symporters. *Nat. Struct. Mol. Biol.* **17**, 822 (2010).
36. Shimamura, T. *et al.* Molecular Basis of Alternating Access Membrane Transport by the Sodium-Hydantoin Transporter, Mhp1. *Science*. **328**, 470

- (2010).
37. Raba, M. *et al.* Extracellular loop 4 of the proline transporter PutP controls the periplasmic entrance to ligand binding sites. *Structure* **22**, 769 (2014).
 38. Bracher, S. *et al.* Glu-311 in external loop 4 of the sodium/proline transporter PutP is crucial for external gate closure. *J. Biol. Chem.* **291**, 4998 (2016).
 39. Loland, C. J. The use of LeuT as a model in elucidating binding sites for substrates and inhibitors in neurotransmitter transporters. *Biochim Biophys Acta Gen Subj* **1850**, 500 (2015).
 40. Yamashita, A., Singh, S. K., Kawate, T., Jin, Y. & Gouaux, E. Crystal structure of a bacterial homologue of Na⁺/Cl⁻-dependent neurotransmitter transporters. *Nature* **437**, 215 (2005).
 41. Kazmier, K. *et al.* Conformational dynamics of ligand-dependent alternating access in LeuT. *Nat. Struct. Mol. Biol.* **21**, 472 (2014).
 42. Faham, S. *et al.* The crystal structure of a sodium galactose transporter reveals mechanistic insights into Na⁺/sugar symport. *Science*. **321**, 810 (2008).
 43. Watanabe, A. *et al.* The mechanism of sodium and substrate release from the binding pocket of vSGLT. *Nature* **468**, 988 (2010).
 44. Paz, A. *et al.* Conformational transitions of the sodium-dependent sugar transporter, vSGLT. *Proc. Natl. Acad. Sci. USA* **115**, e2742 (2018).
 45. Weyand, S. *et al.* Structure and molecular mechanism of a nucleobase-cation-symport-1 family transporter. *Science (80-.).* **322**, 709 (2008).
 46. Kazmier, K. *et al.* Conformational cycle and ion-coupling mechanism of the Na⁺/hydantoin transporter Mhp1. *Proc. Natl. Acad. Sci. USA* **111**, 14752 (2014).
 47. Ressler, S., Terwisscha Van Scheltinga, A. C., Vorrhein, C., Ott, V. & Ziegler, C. Molecular basis of transport and regulation in the Na⁺/betaine symporter BetP. *Nature* **458**, 47 (2009).
 48. Ziegler, C. *et al.* Projection structure and oligomeric state of the osmoregulated sodium/glycine betaine symporter BetP of *Corynebacterium glutamicum*. *J. Mol. Biol.* **337**, 1137 (2004).
 49. Nicklisch, S. C. T. *et al.* Conformational changes of the betaine transporter

- BetP from *Corynebacterium glutamicum* studied by pulse EPR spectroscopy. *Biochim Biophys Acta* **1818**, 359 (2012).
50. Leone, V. *et al.* Interpretation of spectroscopic data using molecular simulations for the secondary active transporter BetP. *J. Gen. Physiol.* **151**, 381 (2019).
 51. Spindler, P. E. *et al.* Carr-Purcell Pulsed Electron Double Resonance with Shaped Inversion Pulses. *J. Phys. Chem. Lett.* **6**, 4331 (2015).
 52. Henderson, P. J. F. The 12-transmembrane helix transporters. *Curr. Opin. Cell Biol.* **5**, 708 (1993).
 53. Marsh, D. & Henderson, P. J. F. Specific spin labelling of the sugar-H⁺ symporter, GalP, in cell membranes of *Escherichia coli*: Site mobility and overall rotational diffusion of the protein. *Biochim Biophys Acta Biomemb* **1510**, 464 (2001).
 54. Hubert, A., Henderson, P. J. F. & Marsh, D. Lipid-protein interactions in *Escherichia coli* membranes overexpressing the sugar-H⁺ symporter, GalP EPR of spin-labelled lipids. *Biochim Biophys Acta Biomemb* **1611**, 243 (2003).
 55. Kumar, H. *et al.* Structure of sugar-bound LacY. *Proc. Natl. Acad. Sci. USA* **111**, 1784 (2014).
 56. Guan, L., Mirza, O., Verner, G., Iwata, S. & Kaback, H. R. Structural determination of wild-type lactose permease. *Proc. Natl. Acad. Sci. USA* **104**, 15294 (2007).
 57. Smirnova, I. *et al.* Sugar binding induces an outward facing conformation of LacY. *Proc. Natl. Acad. Sci. USA* **104**, 16504 (2007).
 58. Smirnova, I. A., Ädelroth, P. & Brzezinski, P. Extraction and liposome reconstitution of membrane proteins with their native lipids without the use of detergents. *Sci. Rep.* **8**, 14950 (2018).
 59. Zhuang, X. & Klauda, J. B. Modeling structural transitions from the periplasmic-open state of lactose permease and interpretations of spin label experiments. *Biochim Biophys Acta Biomemb* **1858**, 1541 (2016).
 60. Fowler, P. W. *et al.* Gating topology of the proton-coupled oligopeptide symporters. *Structure* **23**, 290 (2015).
 61. Newstead, S. *et al.* Crystal structure of a prokaryotic homologue of the

- mammalian oligopeptide-proton symporters, PepT1 and PepT2. *EMBO J.* **30**, 417 (2011).
62. Dvorak, V. *et al.* An Overview of Cell-Based Assay Platforms for the Solute Carrier Family of Transporters. *Front. Pharmacol.* **12**, 722889 (2021).
 63. Aykaç, A. & Şehirli, A. Ö. The Role of the SLC Transporters Protein in the Neurodegenerative Disorders. *Clin. Psychopharmacol. Neurosci.* **18**, 174 (2020).
 64. Purves, D. *et al.* Chemical Synapses. in *Neuroscience* (Sinauer Associates, 2001).
 65. Shigeri, Y., Seal, R. P. & Shimamoto, K. Molecular pharmacology of glutamate transporters, EAATs and VGLUTs. *Brain Res. Rev.* **45**, 250 (2004).
 66. Malik, A. R. & Willnow, T. E. Excitatory amino acid transporters in physiology and disorders of the central nervous system. *Int. J. Mol. Sci.* **20**, 5671 (2019).
 67. Rodríguez-Campuzano, A. G. & Ortega, A. Glutamate transporters: Critical components of glutamatergic transmission. *Neuropharmacology* **192**, 108602 (2021).
 68. Stobart, J. L. & Anderson, C. M. Multifunctional role of astrocytes as gatekeepers of neuronal energy supply. *Front. Cell. Neurosci.* **7**, 38 (2013).
 69. Grewer, C., Gameiro, A. & Rauen, T. SLC1 Glutamate Transporters The SLC1 transporter family. *Pflugers Arch - Eur. J. Phy.* **466**, 3 (2015).
 70. Li, X., Wang, W., Yan, J. & Zeng, F. Glutamic Acid Transporters: Targets for Neuroprotective Therapies in Parkinson's Disease. *Front. Neurosci.* **15**, 678154 (2021).
 71. Divito, C. B. & Underhill, S. M. Excitatory amino acid transporters: Roles in glutamatergic neurotransmission. *Neurochem.Int.* **73**, 172 (2014).
 72. Kanai, Y. *et al.* The SLC1 high-affinity glutamate and neutral amino acid transporter family. *Mol. Asp. Med.* **34**, 108 (2013).
 73. Pines, G. *et al.* Cloning and expression of a rat brain L-glutamate transporter. *Nature* **360**, 464 (1992).
 74. Kanai, Y. & Hediger, M. A. Primary structure and functional characterization of a high-affinity glutamate transporter. *Nature* **360**, 467 (1992).
 75. Storck, T., Schulte, S., Hofmann, K. & Stoffel, W. Structure, expression, and

- functional analysis of a Na⁺-dependent glutamate/aspartate transporter from rat brain. *Proc. Natl. Acad. Sci. USA* **89**, 10955 (1992).
76. Arriza, J. L. *et al.* Functional Comparisons of Three Glutamate Cloned from Human Motor Cortex Transporter. *J. Neurosci.* **14**, 5559 (1994).
 77. Fairman, W. A., Vandenberg, R. J., Arriza, J. L., Kavanaugh, M. P. & Amara, S. G. An excitatory amino-acid transporter with properties of a ligand-gated chloride channel. *Nature* **246**, 170 (1973).
 78. Arriza, J. L., Eliasof, S., Kavanaugh, M. P. & Amara, S. G. Excitatory amino acid transporter 5, a retinal glutamate transporter coupled to a chloride conductance. *Proc. Natl. Acad. Sci. USA* **94**, 4155 (1997).
 79. Deng, X. *et al.* Association study of polymorphisms in the glutamate transporter genes SLC1A1, SLC1A3, and SLC1A6 with schizophrenia. *Am. J. Med. Genet. B Neuropsychiatr. Genet.* **144**, 271 (2007).
 80. Li, S., Mallory, M., Alford, M., Tanaka, S. & Masliah, E. Glutamate transporter alterations in Alzheimer disease are possibly associated with abnormal AAP expression. *J. Neuropathol. Exp. Neurol.* **56**, 901 (1997).
 81. Dadas, A. & Janigro, D. Breakdown of blood brain barrier as a mechanism of post-traumatic epilepsy. *Neurobiol. Dis.* **123**, 20 (2019).
 82. Arranz, A. M., Gottlieb, M., Pérez-Cerdá, F. & Matute, C. Increased expression of glutamate transporters in subcortical white matter after transient focal cerebral ischemia. *Neurobiol. Dis.* **37**, 156 (2010).
 83. Canul-Tec, J. C. *et al.* Structure and allosteric inhibition of excitatory amino acid transporter 1. *Nature* **544**, 446 (2017).
 84. Qiu, B. & Boudker, O. Symport and antiport mechanisms of human glutamate transporters. *Nat Commun.* **14**, 2579 (2023).
 85. Zhang, Z. *et al.* Structural basis of ligand binding modes of human EAAT2. *Nat Commun.* **13**, 3329 (2022).
 86. Kato, T. *et al.* Structural insights into inhibitory mechanism of human excitatory amino acid transporter EAAT2. *Nat Commun.* **13**, 4714 (2022).
 87. Canul-Tec, J. C. *et al.* The ion-coupling mechanism of human excitatory amino acid transporters. *EMBO J.* **41**, e108341 (2022).
 88. Qiu, B., Matthies, D., Fortea, E., Yu, Z. & Boudker, O. Cryo-EM structures of

- excitatory amino acid transporter 3 visualize coupled substrate , sodium , and proton binding and transport. *Sci Adv.* **7**, eabf5814 (2021).
89. Kanner, B. I. & Sharon, I. Active transport of L-glutamate by membrane vesicles isolated from rat brain. *Biochim Biophys Acta Biomemb* **17**, 3949 (1978).
 90. Barbour, B., Brew, H. & Attwell, D. Electrogenic glutamate uptake in glial cells is activated by intracellular potassium. *Nature* **335**, 433 (1988).
 91. Brew, H. & Attwell, D. Electrogenic glutamate uptake is a major current carrier in the membrane of axolotl retinal glial cells. *Nature* **327**, 707 (1987).
 92. Grewer, C., Watzke, N., Rauen, T. & Bicho, A. Is the glutamate residue Glu-373 the proton acceptor of the excitatory amino acid carrier 1? *J. Biol. Chem.* **278**, 2585 (2003).
 93. Watzke, N., Rauen, T., Bamberg, E. & Grewer, C. On the mechanism of proton transport by the neuronal excitatory amino acid carrier 1. *J. Gen. Physiol.* **116**, 609 (2000).
 94. Vandenberg, R. J. & Ryan, R. M. Mechanisms of glutamate transport. *Physiol Rev* **93**, 1621 (2013).
 95. Zerangue, N. & Kavanaugh, M. P. Flux coupling in a neuronal glutamate transporter. *Nature* **383**, 634 (1996).
 96. Wersinger, E. *et al.* The glutamate transporter EAAT5 works as a presynaptic receptor in mouse rod bipolar cells. *J. Physiol.* **577**, 221 (2006).
 97. Veruki, M. L., Mørkve, S. H. & Hartveit, E. Activation of a presynaptic glutamate transporter regulates synaptic transmission through electrical signaling. *Nat Neurosci* **9**, 1388 (2006).
 98. Drew, D. & Boudker, O. Shared Molecular Mechanisms of Membrane Transporters. *Annu. Rev. Biochem.* **85**, 543 (2016).
 99. Drew, D., North, R. A., Nagarathinam, K. & Tanabe, M. Structures and General Transport Mechanisms by the Major Facilitator Superfamily (MFS). *Chem. Rev.* **121**, 5289 (2021).
 100. Kazmier, K., Claxton, D. P. & Mchaourab, H. S. Alternating access mechanisms of LeuT-fold transporters: trailblazing towards the promised energy landscapes. *Curr. Opin. Struct. Biol.* **45**, 100 (2017).

101. Garaeva, A. A. & Slotboom, D. J. Elevator-type mechanisms of membrane transport. *Biochem. Soc. Trans.* **48**, 1227 (2020).
102. Reyes, N., Ginter, C. & Boudker, O. Transport mechanism of a bacterial homologue of glutamate transporters. *Nature* **462**, 880 (2009).
103. Erkens, G. B., Hänel, I., Goudsmits, J. M. H., Slotboom, D. J. & Van Oijen, A. M. Unsynchronised subunit motion in single trimeric sodium-coupled aspartate transporters. *Nature* **502**, 119 (2013).
104. Ruan, Y. *et al.* Direct visualization of glutamate transporter elevator mechanism by high-speed AFM. *Proc. Natl. Acad. Sci. USA* **114**, 1584 (2017).
105. Akyuz, N., Altman, R. B., Blanchard, S. C. & Boudker, O. Transport dynamics in a glutamate transporter homologue. *Nature* **502**, 114 (2013).
106. Grewer, C. *et al.* Individual subunits of the glutamate transporter EAAC1 homotrimer function independently of each other. *Biochemistry* **44**, 11913 (2005).
107. Stolzenberg, S., Khelashvili, G. & Weinstein, H. Structural intermediates in a model of the substrate translocation path of the bacterial glutamate transporter homologue GltPh. *J. Phys. Chem. B* **116**, 5372 (2012).
108. Yernool, D., Boudker, O., Jin, Y. & Gouaux, E. Structure of a glutamate transporter homologue from *Pyrococcus horikoshii*. *Nature* **431**, 811 (2004).
109. Jensen, S., Guskov, A., Rempel, S., Hänel, I. & Slotboom, D. J. Crystal structure of a substrate-free aspartate transporter. *Nat. Struct. Mol. Biol.* **20**, 1224 (2013).
110. Arkhipova, V., Guskov, A. & Slotboom, D. J. Analysis of the quality of crystallographic data and the limitations of structural models. *J. Gen. Physiol.* **149**, 1091 (2017).
111. Boudker, O., Ryan, R. M., Yernool, D., Shimamoto, K. & Gouaux, E. Coupling substrate and ion binding to extracellular gate of a sodium-dependent aspartate transporter. *Nature* **445**, 387 (2007).
112. Silverstein, N., Ewers, D., Forrest, L. R., Fahlke, C. & Kanner, B. I. Molecular determinants of substrate specificity in sodium-coupled glutamate transporters. *J. Biol. Chem.* **290**, 28988 (2015).
113. Arkhipova, V. *et al.* Binding and transport of D-aspartate by the glutamate

- transporter homolog GltTk. *eLife* **8**, e45286 (2019).
114. Groeneveld, M. & Slotboom, D. J. Na⁺:Aspartate coupling stoichiometry in the glutamate transporter homologue GltPh. *Biochemistry* **49**, 3511 (2010).
 115. Ryan, R. M. & Mindell, J. A. The uncoupled chloride conductance of a bacterial glutamate transporter homolog. *Nat Struct Mol Biol* **14**, 365 (2007).
 116. Guskov, A., Jensen, S., Faustino, I., Marrink, S. J. & Slotboom, D. J. Coupled binding mechanism of three sodium ions and aspartate in the glutamate transporter homologue GltTk. *Nat. Commun.* **7**, 13420 (2016).
 117. Chen, I. *et al.* Glutamate transporters have a chloride channel with two hydrophobic gates. *Nature* **591**, 327 (2021).
 118. Cater, R. J., Vandenberg, R. J. & Ryan, R. M. The Domain Interface of the Human Glutamate Transporter EAAT1 Mediates Chloride Permeation. *Biophys. J.* **107**, 621 (2014).
 119. Colucci, E. *et al.* Mutation in glutamate transporter homologue GltTk provides insights into pathologic mechanism of episodic ataxia 6. *Nat Commun.* **14**, 1799 (2023).
 120. Garaeva, A. A., Guskov, A., Slotboom, D. J. & Paulino, C. A one-gate elevator mechanism for the human neutral amino acid transporter ASCT2. *Nat Commun.* **10**, 3427 (2019).
 121. Wang, X. & Boudker, O. Large domain movements through the lipid bilayer mediate substrate release and inhibition of glutamate transporters. *eLife* **9**, e58417 (2020).
 122. Arkhipova, V., Guskov, A. & Slotboom, D. J. Structural ensemble of a glutamate transporter homologue in lipid nanodisc environment. *Nat Commun.* **11**, 998 (2020).
 123. Bastug, T. *et al.* Position of the third Na⁺ site in the aspartate transporter GltPh and the human glutamate transporter, EAAT1. *PLoS One* **7**, e33058 (2012).
 124. Reyes, N., Oh, S. & Boudker, O. Binding thermodynamics of a glutamate transporter homolog. *Nat Struct Mol Biol* **20**, 634 (2013).
 125. Oh, S. & Boudker, O. Kinetic mechanism of coupled binding in sodium-aspartate symporter GltPh. *eLife* **7**, e37291 (2018).

126. Verdon, G., Oh, S., Serio, R. & Boudker, O. Coupled ion binding and structural transitions along the transport cycle of glutamate transporters. *eLife* **2014**, e02283 (2014).
127. Bendahan, A., Armon, A., Madani, N., Kavanaugh, M. P. & Kanner, B. I. Arginine 447 plays a pivotal role in substrate interactions in a neuronal glutamate transporter. *J. Biol. Chem.* **275**, 37436 (2000).
128. Hänelt, I., Jensen, S., Wunnicke, D. & Slotboom, D. J. Low affinity and slow Na⁺ binding precedes high affinity aspartate binding in the secondary-active transporter GltPh. *J. Biol. Chem.* **290**, 15962 (2015).
129. Akyuz, N. *et al.* Transport domain unlocking sets the uptake rate of an aspartate transporter. *Nature* **518**, 68 (2015).
130. Cater, R. J., Ryan, R. M. & Vandenberg, R. J. The Split Personality of Glutamate Transporters: A Chloride Channel and a Transporter. *Neurochem. Res.* **41**, 593 (2016).
131. Pavic, A., Holmes, A. O. M., Postis, V. L. G. & Goldman, A. Glutamate transporters: A broad review of the most recent archaeal and human structures. *Biochem. Soc. Trans.* **47**, 1197 (2019).
132. Slotboom, D. J., Sobczak, I., Konings, W. N. & Lolkema, J. S. A conserved serine-rich stretch in the glutamate transporter family forms a substrate-sensitive reentrant loop. *Proc. Natl. Acad. Sci. USA* **96**, 14282 (1999).
133. Hall, J. L. *et al.* Saturation transfer difference NMR on the integral trimeric membrane transport protein GltPh determines cooperative substrate binding. *Sci. Rep.* **10**, 16483 (2020).
134. Georgieva, E. R., Borbat, P. P., Ginter, C., Freed, J. H. & Boudker, O. Conformational ensemble of the sodium-coupled aspartate transporter. *Nat. Struct. Mol. Biol.* **20**, 215 (2013).
135. Hänelt, I., Wunnicke, D., Bordignon, E., Steinhoff, H.-J. & Slotboom, D. J. Conformational heterogeneity of the aspartate transporter GltPh. *Nat. Struct. Mol. Biol.* **20**, 210 (2013).
136. Ciftci, D. *et al.* Single-molecule transport kinetics of a glutamate transporter homolog shows static disorder. *Sci. Adv.* **6**, eaaz1949 (2020).
137. Grewer, C., Watzke, N., Wiessner, M. & Rauen, T. Glutamate translocation of

- the neuronal glutamate transporter EAAC1 occurs within milliseconds. *Proc. Natl. Acad. Sci. USA* **97**, 9706 (2000).
138. Larsson, H. P., Tzingounis, A. V., Koch, H. P. & Kavanaugh, M. P. Fluorometric measurements of conformational changes in glutamate transporters. *Proc. Natl. Acad. Sci. USA* **101**, 3951 (2004).
 139. Huysmans, G. H. M., Ciftci, D., Wang, X., Blanchard, S. C. & Boudker, O. The high-energy transition state of the glutamate transporter homologue GltPh. *EMBO J.* **40**, e105415 (2021).
 140. Zhou, W., Trinco, G., Slotboom, D. J., Forrest, L. R. & Faraldo-Gómez, J. D. On the Role of a Conserved Methionine in the Na⁺-Coupling Mechanism of a Neurotransmitter Transporter Homolog. *Neurochem. Res.* **47**, 163 (2022).
 141. Trinco, G. *et al.* Kinetic mechanism of Na⁺-coupled aspartate transport catalyzed by GltTk. *Commun. Biol.* **4**, 751 (2021).
 142. Focke, P. J., Moenne-Loccoz, P. & Larsson, H. P. Opposite movement of the external gate of a glutamate transporter homolog upon binding cotransported sodium compared with substrate. *J. Neurosci.* **31**, 6255 (2011).
 143. Ryan, R. M., Compton, E. L. R. & Mindell, J. A. Functional characterization of a Na⁺-dependent aspartate transporter from *Pyrococcus horikoshii*. *J. Biol.* **284**, 17540 (2009).
 144. Riederer, E. A. *et al.* A facile approach for the in vitro assembly of multimeric membrane transport proteins. *eLife* **7**, e36478 (2018).
 145. Riederer, E. A., Moënné-Loccoz, P. & Valiyaveetil, F. I. Distinct roles of the Na⁺ binding sites in the allosteric coupling mechanism of the glutamate transporter homolog, GltPh. *Proc. Natl. Acad. Sci. USA* **119**, e2121653119 (2022).
 146. Huang, Y. *et al.* Use of Paramagnetic ¹⁹F NMR to Monitor Domain Movement in a Glutamate Transporter. *Nat. Chem. Biol.* **16**, 1006 (2020).
 147. Huang, Z. & Tajkhorshid, E. Identification of the third Na⁺ site and the sequence of extracellular binding events in the glutamate transporter. *Biophys. J.* **99**, 1416 (2010).
 148. Alleva, C. *et al.* Na⁺-dependent gate dynamics and electrostatic attraction ensure substrate coupling in glutamate transporters. *Sci Adv.* **6**, eaba9854

- (2020).
149. Zhou, W. *et al.* Large-scale state-dependent membrane remodeling by a transporter protein. *eLife* **8**, e50576 (2019).
 150. Chechik, V., Carter, E. & Murphy, D. *Electron Paramagnetic Resonance*. (Oxford University Press, 2016).
 151. Brustolon, M. & Giamello, E. *Electron Paramagnetic Resonance: A Practitioner's Toolkit*. (John Wiley & Sons, 2009).
 152. Eaton, G., Eaton, S., Barr, D. & Weber, R. *Quantitative EPR*. (Springer-Verlag, 2010).
 153. Weber, R., Jiang, J. & Barr, D. *An EPR Primer*. (Bruker Instruments Inc, 1998).
 154. Sahu, I. D. & Lorigan, G. A. Electron Paramagnetic Resonance as a Tool for Studying Membrane Proteins. *Biomolecules* **10**, 763 (2020).
 155. Torricella, F., Pierro, A., Mileo, E., Belle, V. & Bonucci, A. Nitroxide spin labels and EPR spectroscopy: A powerful association for protein dynamics studies. *BBA - Proteins Proteomics* **1869**, 140653 (2021).
 156. Fleissner, M. R. *et al.* Site-directed spin labeling of a genetically encoded unnatural amino acid. *Proc. Natl. Acad. Sci. USA* **106**, 21637 (2009).
 157. Weil, J. & Bolton, J. *Electron Paramagnetic Resonance*. (John Wiley & Sons, 2006).
 158. Abdullin, D. *et al.* Pulsed EPR Dipolar Spectroscopy under the Breakdown of the High-Field Approximation: The High-Spin Iron(III) Case. *Chem. Eur. J.* **25**, 8820 (2019).
 159. Owenius, R., Engström, M., Lindgren, M. & Huber, M. Influence of solvent polarity and hydrogen bonding on the EPR parameters of a nitroxide spin label studied by 9-GHz and 95-GHz EPR spectroscopy and DFT calculations. *J. Phys. Chem. A* **105**, 10967 (2001).
 160. Stoll, S. Pulse EPR. *eMagRes* **6**, 23 (2017).
 161. Weber, R. *Pulsed EPR Primer*. (Bruker Instruments Inc, 2000).
 162. Goldfarb, D. & Stoll, S. *EPR Spectroscopy: Fundamentals and Methods*. (John Wiley & Sons, 2018).
 163. Bahrenberg, T., Jahn, S. M., Feintuch, A., Stoll, S. & Goldfarb, D. The decay of the refocused Hahn echo in double electron-electron resonance (DEER)

- experiments. *Magn Reson.* **2**, 161 (2021).
164. Jeschke, G. *et al.* DeerAnalysis2006 - A Comprehensive Software Package for Analyzing Pulsed ELDOR data. *Appl. Magn. Res.* **30**, 473 (2006).
 165. Hemminga, M. & Berliner, L. *ESR Spectroscopy in Membrane Biophysics*. (Springer US, 2007).
 166. Jeschke, G. DEER Distance Measurements on Proteins. *Annu. Rev. Phys. Chem.* **63**, 419 (2012).
 167. Reginsson, G. W. & Schiemann, O. Pulsed electron–electron double resonance: beyond nanometre distance measurements on biomacromolecules. *Biochem. J.* **434**, 353 (2011).
 168. Worswick, S. G., Spencer, J. A., Jeschke, G. & Kuprov, I. Deep neural network processing of DEER data. *Sci Adv.* **4**, eaat5218 (2018).
 169. Ibáñez, L. F., Jeschke, G. & Stoll, S. The new DeerAnalysis toolbox for analyzing dipolar EPR spectroscopy data. *Magn Reson.* **1**, 209 (2020).
 170. Sugiki, T., Furuita, K., Fujiwara, T. & Kojima, C. Current NMR techniques for structure-based drug discovery. *Molecules* **23**, 148 (2018).
 171. Teng, Q. *Structural biology: Practical NMR applications*. (Springer, 2013).
 172. Viegas, A., Manso, J., Nobrega, F. L. & Cabrita, E. J. Saturation-Transfer Difference (STD) NMR: A Simple and Fast Method for Ligand Screening and Characterization of Protein Binding. *J. Chem. Edu.* **88**, 990 (2011).
 173. Liang, B. & Tamm, L. K. NMR as a Tool to Investigate Membrane Protein Structure, Dynamics and Function. *Nat Struct Mol Biol* **23**, 468 (2016).
 174. Markwick, P. R. L., Malliavin, T. & Nilges, M. Structural biology by NMR: Structure, dynamics, and interactions. *PLoS Comput. Biol.* **4**, e1000168 (2008).
 175. Tugarinov, V., Muhandiram, R., Ayed, A. & Kay, L. E. Four-dimensional NMR spectroscopy of a 723-residue protein: Chemical shift assignments and secondary structure of malate synthase G. *J. Am. Chem. Soc* **124**, 10025 (2002).
 176. Nygaard, R., Kim, J. & Mancina, F. Cryo-electron microscopy analysis of small membrane proteins. *Curr. Opin. Struct. Biol.* **64**, 26 (2020).
 177. Kay, L. E. NMR studies of protein structure and dynamics. *J. Mag. Res.* **173**,

- 193 (2005).
178. Foster, M. P., McElroy, C. A. & Amero, C. D. Solution NMR of large molecules and assemblies. *Biochemistry* **46**, 331 (2007).
179. Meyer, B. & Peters, T. NMR Spectroscopy Techniques for Screening and Identifying Ligand Binding to Protein Receptors. *Angew. Chem. Int. Ed.* **42**, 864 (2003).
180. Angulo, J., Enríquez-Navas, P. M. & Nieto, P. M. Ligand-receptor binding affinities from saturation transfer difference (STD) NMR. *Chem. Eur. J.* **16**, 7803 (2010).
181. Monaco, S. *et al.* Exploring Multi-Subsite Binding Pockets in Proteins: DEEP-STD NMR Fingerprinting and Molecular Dynamics Unveil a Cryptic Subsite at the GM1 Binding Pocket of Cholera Toxin B. *Chem. Eur. J.* **26**, 10024 (2020).
182. Venkatesan, S. K. *et al.* Refinement of the Central Steps of Substrate Transport by the Aspartate Transporter GltPh: Elucidating the Role of the Na² Sodium Binding Site. *PLoS Comput. Biol.* **11**, e1004551 (2015).
183. Jeschke, G. MMM: A toolbox for integrative structure modeling. *Protein Sci.* **27**, 76 (2018).
184. Arkhipova, V. *et al.* Structural Aspects of Photopharmacology: Insight into the Binding of Photoswitchable and Photocaged Inhibitors to the Glutamate Transporter Homologue. *J. Am. Chem. Soc.* **143**, 1513 (2021).
185. Riederer, E. A. & Valiyaveetil, F. I. Investigation of the allosteric coupling mechanism in a glutamate transporter homolog via unnatural amino acid mutagenesis. *Proc. Natl. Acad. Sci. USA* **116**, 15939 (2019).
186. Georgieva, E. R. *et al.* Effect of freezing conditions on distances and their distributions derived from Double Electron Electron Resonance (DEER): A study of doubly-spin-labeled T4 lysozyme. *J. Mag. Res.* **216**, 69 (2012).
187. Wort, J. L., Ackermann, K., Norman, D. G. & Bode, B. E. A general model to optimise Cu(II) labelling efficiency of double-histidine motifs for pulse dipolar EPR applications. *Phys. Chem. Chem. Phys.* **23**, 3810 (2021).
188. Ghosh, S., Saxena, S. & Jeschke, G. Rotamer Modelling of Cu(II) Spin Labels Based on the Double-Histidine Motif. *Appl. Magn. Res.* **49**, 1281 (2018).
189. Cunningham, T. F., Putterman, M. R., Desai, A., Horne, W. S. & Saxena, S. The

- Double-Histidine Cu²⁺-Binding Motif: A Highly Rigid, Site-Specific Spin Probe for Electron Spin Resonance Distance Measurements. *Angew. Chem. Int. Ed. Engl.* **54**, 6330 (2015).
190. Lawless, M. J., Ghosh, S., Cunningham, T. F., Shimshi, A. & Saxena, S. On the use of the Cu²⁺-iminodiacetic acid complex for double histidine based distance measurements by pulsed ESR. *Phys. chem. chem. phys.* **19**, 20959 (2017).
 191. Gamble Jarvi, A., Casto, J. & Saxena, S. Buffer effects on site directed Cu²⁺-labeling using the double histidine motif. *J. Mag. Res.* **320**, 106848 (2020).
 192. Van Wonderen, J. H., Kostrz, D. N., Dennison, C. & MacMillan, F. Refined distances between paramagnetic centers of a multi-copper nitrite reductase determined by pulsed EPR (iDEER) spectroscopy. *Angew. Chem. Int. Ed.* **52**, 1990 (2013).
 193. Peisach, J. & Blumberg, W. E. Structural implications derived from the analysis of electron paramagnetic resonance spectra of natural and artificial copper proteins. *Arch. Biochem.* **165**, 691 (1974).
 194. Wort, J. L. *et al.* Sub-Micromolar Pulse Dipolar EPR Spectroscopy Reveals Increasing CuII-labelling of Double-Histidine Motifs with Lower Temperature. *Angew. Chem. Int. Ed.* **58**, 11681 (2019).
 195. Milikisyants, S., Scarpelli, F., Finiguerra, M. G., Ubbink, M. & Huber, M. A pulsed EPR method to determine distances between paramagnetic centers with strong spectral anisotropy and radicals: The dead-time free RIDME sequence. *J. Mag. Res.* **201**, 48 (2009).
 196. Steinhoff, H.-J. *et al.* Determination of interspin distances between spin labels attached to insulin: Comparison of electron paramagnetic resonance data with the X-ray structure. *Biophys. J.* **73**, 3287 (1997).
 197. Mehlenbacher, M. R., Bou-Abdallah, F., Liu, X. X. & Melman, A. Calorimetric studies of ternary complexes of Ni(II) and Cu(II) nitrilotriacetic acid and N-acetyloligohistidines. *Inorganica Chim. Acta* **437**, 152 (2015).
 198. Harju, L. & Sara, R. Spectrophotometric titrations of nitrilotriacetic acid with copper(II) as monoligand and biligand chelates. *Anal. Chim. Acta* **73**, 129 (1974).

199. Ghosh, S., Lawless, M. J., Rule, G. S. & Saxena, S. The Cu²⁺-nitrilotriacetic acid complex improves loading of α -helical double histidine site for precise distance measurements by pulsed ESR. *J. Mag. Res.* **286**, 163 (2018).
200. Reddy, K. D., Ciftci, D., Scopelliti, A. J. & Boudker, O. The archaeal glutamate transporter homologue GltPh shows heterogeneous substrate binding. *J. Gen. Physiol.* **154**, e202213131 (2022).
201. Boudker, O. & Oh, S. Isothermal titration calorimetry of ion-coupled membrane transporters. *Methods* **76**, 171 (2015).
202. Wegener, H. *et al.* Human norovirus GII.4(MI001) P dimer binds fucosylated and sialylated carbohydrates. *Glycobiology* **27**, 1027 (2017).
203. Ritsch, I., Hintz, H., Jeschke, G., Godt, A. & Yulikov, M. Improving the accuracy of Cu(II)-nitroxide RIDME in the presence of orientation correlation in water-soluble Cu(II)-nitroxide rulers. *Phys. chem. chem. phys.* **21**, 9810 (2019).
204. Chang, A. Y., Chau, V. W. Y., Landas, J. A. & Pang, Y. Preparation of calcium competent *Escherichia coli* and heat-shock transformation. *J. Exp. Microbiol. Immunol.* **1**, 22 (2017).
205. Wu, C. H., Bialecka-Fornal, M. & Newman, D. K. Methylation at the C-2 position of hopanoids increases rigidity in native bacterial membranes. *eLife* **2015**, e05663 (2015).
206. Regan, D., Williams, J., Borri, P. & Langbein, W. Lipid Bilayer Thickness Measured by Quantitative DIC Reveals Phase Transitions and Effects of Substrate Hydrophilicity. *Langmuir* **35**, 13805 (2019).
207. Bye, M. An efficient EPR method for characterising protein-protein interactions. (University of East Anglia, 2013).
208. Pannier, M., Veit, S., Godt, A., Jeschke, G. & Spiess, H. W. Dead-Time Free Measurement of Dipole-Dipole Interactions between Electron Spins. *J. Mag. Res.* **142**, 331 (2000).
209. Stoll, S. & Schweiger, A. EasySpin, a comprehensive software package for spectral simulation and analysis in EPR. *J. Mag. Res.* **178**, 42 (2006).

Appendix

A1 K_D values of Glt_{Tk} and Glt_{Ph} for L-glutamate and L-aspartate

Protein	Technique	Substrate	[NaCl] / mM	[substrate] / μ M
Glt _{Ph} ¹³³	STD-NMR	L-glutamate	100	414 μ M 1034 μ M*
Glt _{Ph} L130W ¹¹²	Fluorescence-based assay	L-glutamate	200	237 μ M*

Protein	Technique	Substrate	[NaCl] / mM	[substrate] / nM
Glt _{Ph} ¹³³	STD-NMR	L-aspartate	100	42 13 *
Glt _{Ph} L130W ¹¹¹	Fluorescence-based assay	L-aspartate	200	1 *
Glt _{Ph} ¹²⁶	ITC	L-aspartate	100	27 **
Glt _{Ph} L130W ¹¹²	Fluorescence-based assay	L-aspartate	200	4.9 *
Glt _{Ph} ¹²⁹	ITC	L-aspartate	10	380
Glt _{Ph} L130W ¹²⁸	ITC	L-aspartate	10	512
			200	1.5
	Steady state fluorescence	L-aspartate	10	250
			200	2.6
Glt _{Ph} ¹²⁴	ITC	L-aspartate	10	480
Glt _{Ph} ¹⁸²	Uptake	L-aspartate	100	119 *
Glt _{Tk} ¹¹³	ITC	L-aspartate	500	62
			300	120
			75	1040

Table A1 A summary of the dissociation constants for Glt_{Tk} and Glt_{Ph} with either L-aspartate or L-glutamate. The technique and sodium concentration used are included with corresponding references; Protein in detergent micelles unless signified by* to show reconstitution into proteoliposomes. **binding was too tight to be accurately measured.

A2 Glt_{Tk} uptake assay and ITC results (by Emanuela Colucci and Zaid Anshari)

Collaborators at the University of Groningen tested Glt_{Tk} samples produced and purified in house using uptake assays to test their activity. All results were compared to those of wild type (WT) Glt_{Tk}. For sodium dependence studies, at concentrations ranging between 0–150 mM NaCl, it was found that Glt_{Tk} I312C had slightly gained activity in comparison to WT. The other variants lost activity to varying extents, with Glt_{Tk} S281C showing very low activity (Figure A1). In time course studies, S281C again showed the biggest drop in activity with I312C being comparable to that of WT Glt_{Tk} (Figure A2). Given the most promising EPR results were for Glt_{Tk} S281C, this variant was further investigated as labelled vs unlabelled protein (Figure A3). It was concluded that both samples of Glt_{Tk} are incapable, or very inefficient, at transporting aspartate. Further testing of binding activity using isothermal titration calorimetry (ITC) revealed the K_D values determined for both samples were comparable to WT Glt_{Tk}, with labelled Glt_{Tk} S281C having a higher affinity than the unlabelled protein (Figure A4). The respective K_D values were 67.5 nM (Glt_{Tk} S281C) and 253.4 nM (Glt_{Tk} S281C + MTSL). Glt_{Tk} H334C and D298C were acquired after these studies so their activity was tested using ITC (Figure A5). The K_D values obtained are comparable to those of WT Glt_{Tk}: 68 nM (for D298C), 78 nM (for H334C) and 77 nM (WT).

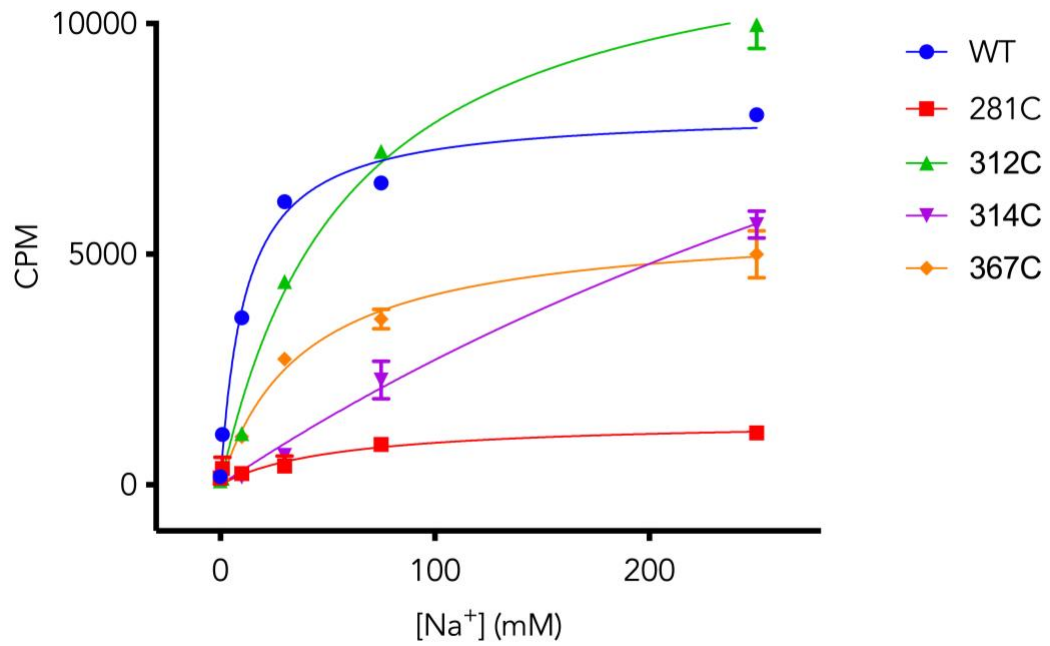


Figure A1 Sodium ion dependency of Glt_{Tk} variants compared to WT using uptake assays. Data was recorded and plotted by Emanuela Colucci (University of Groningen)

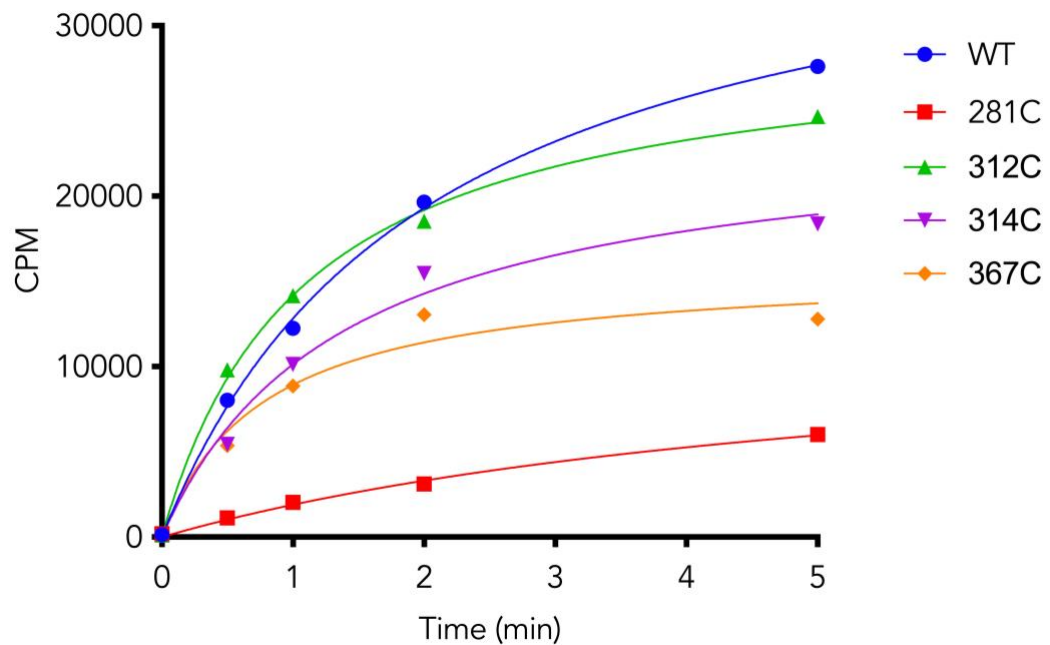


Figure A2 Time course experiments of Glt_{Tk} variants compared to WT using uptake assays. Data was recorded and plotted by Emanuela Colucci (University of Groningen)

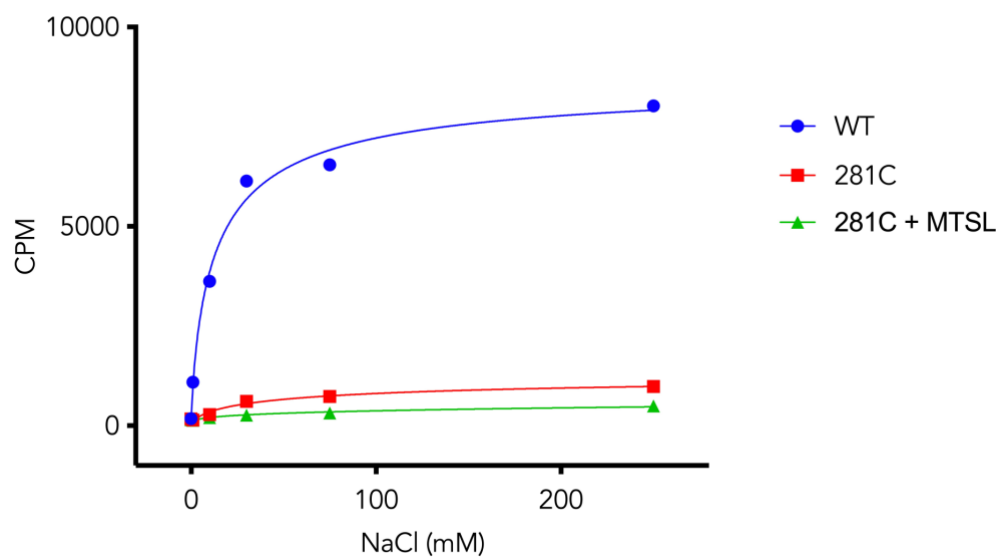


Figure A3 Comparison of Glt_{TK} S281C with and without spin label (MTSL) using uptake assays. Data was recorded and plotted by Emanuela Colucci (University of Groningen)

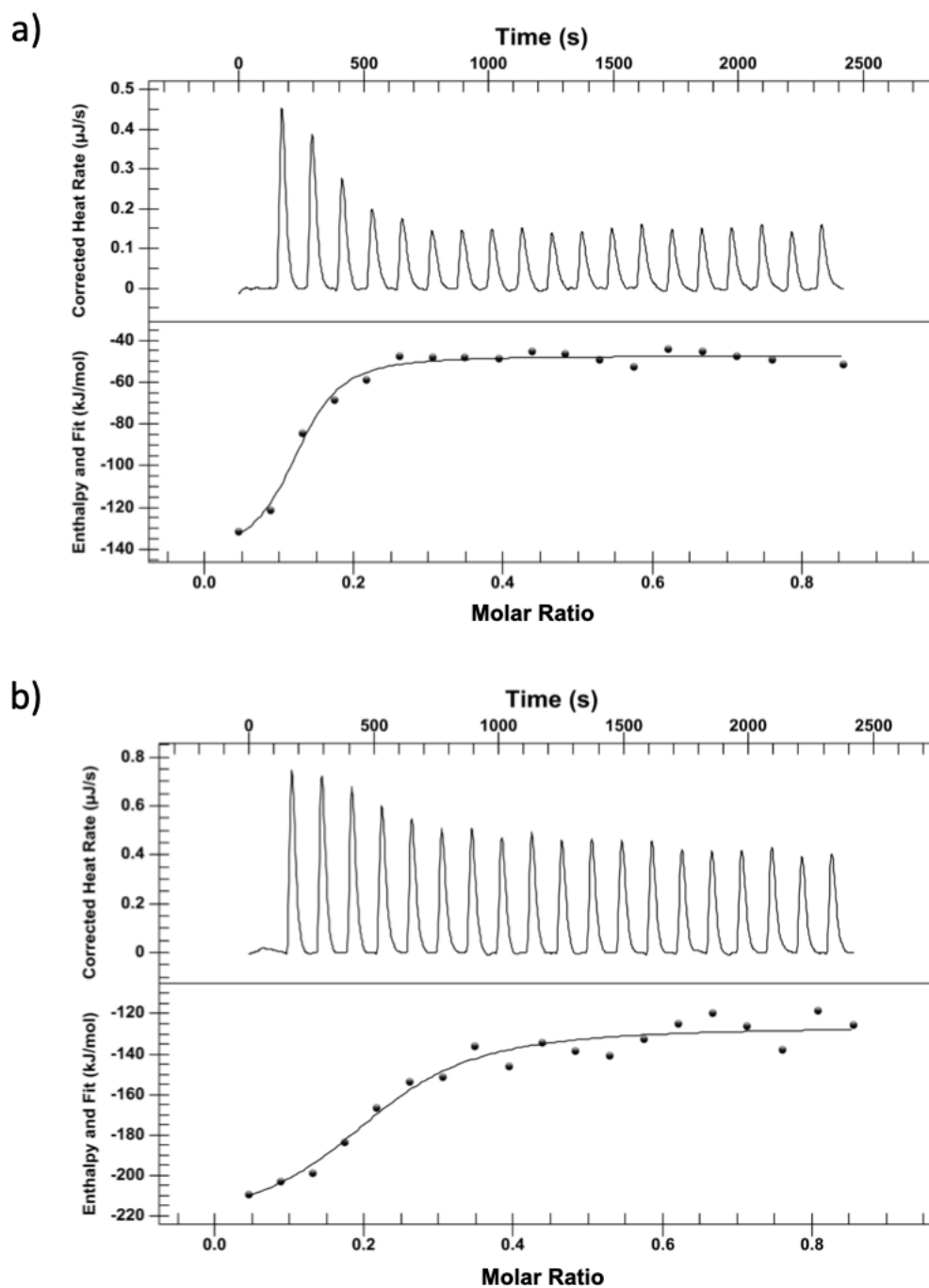


Figure A4 Isothermal titration calorimetry (ITC) results for Glt_{Tk} S281C with and without spin-label. Unlabelled Glt_{Tk} S281C (a) gave a K_D value of 67.5 nM, and for labelled Glt_{Tk} S281C (b) a K_D value of 253.4 nM. Data was recorded and plotted by Emanuela Colucci (University of Groningen)

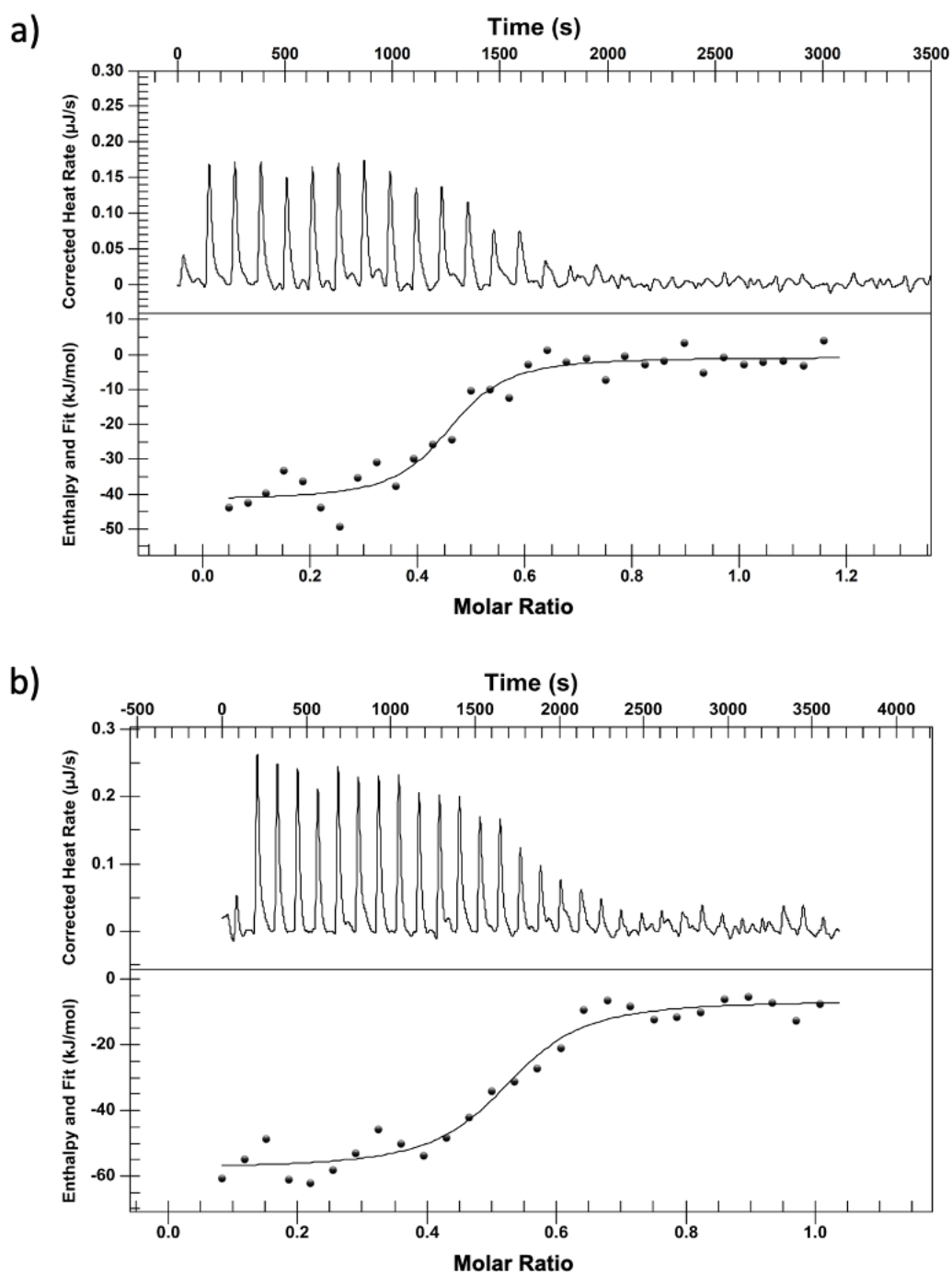


Figure A5 Isothermal titration calorimetry (ITC) results for a) Glt_{7k} D298C and b) H334C. K_D values of 68 nM and 78 nM were calculated respectively. Data was recorded and plotted by Zaid Anshari (University of Groningen)

A3 Sequence alignment of GltTk, GltPh and EAAT3

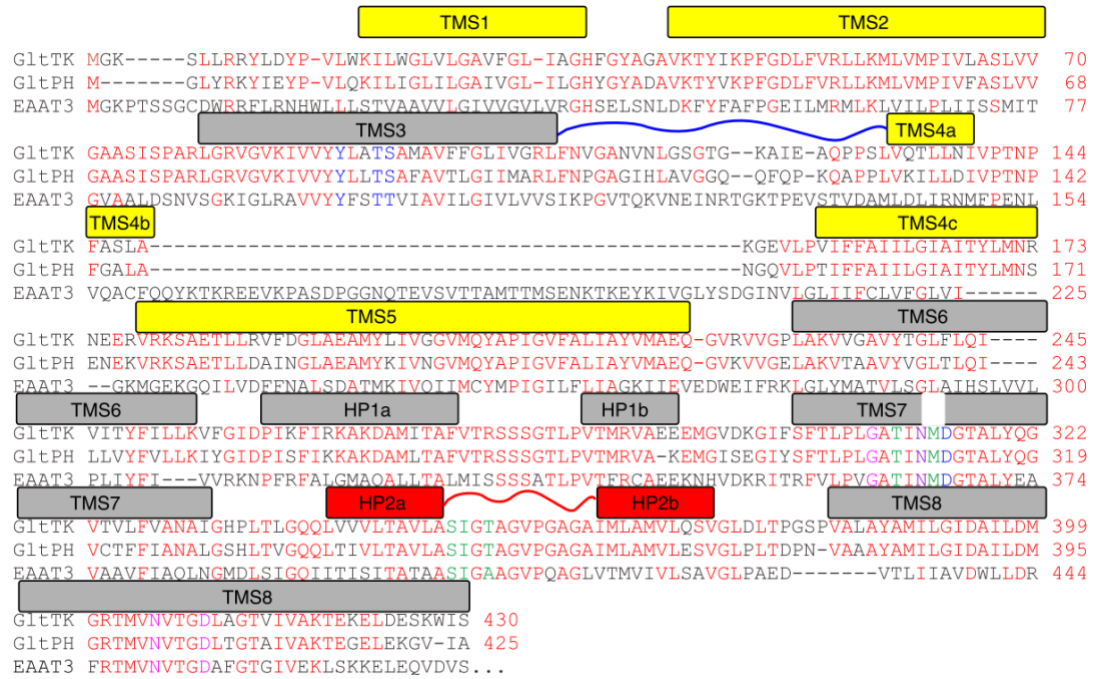


Figure A6 Sequence alignment of the aspartate transporters Glt_{Tk} from *Thermococcus kodakarensis* and Glt_{Ph} from *Pyrococcus horikoshii*; and the glutamate transporter EAAT3 from *Rattus norvegicus*. Identical residues are in red, Na1 binding in magenta, Na2 binding in green and Na3 in blue. Transport domains are shown in grey with scaffold trimerization domain in yellow; with HP2 in red and loop 3-4 in blue. Figure from Guskov et al.¹¹⁶

A4 Glt_{Tk} MMM results visualised as chimera images

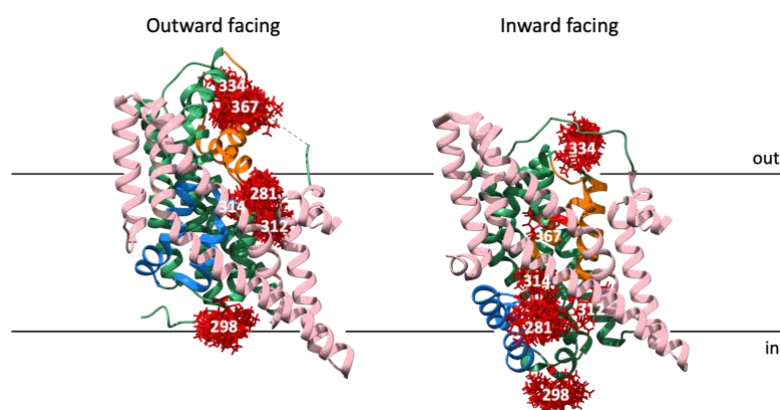


Figure A7 Position of GltTk variants as a chimera structure. Glt_{Tk} is composed of a transport domain (green) and scaffold domain (pink), with HP1 (blue) and HP2 (orange) highlighted. A Glt_{Tk} protomer in an outward open state (left, PDB 4KY0) and inward open (right, PDB 6XWR). Red clusters represent attachment of spin-label using *in silico* modelling.

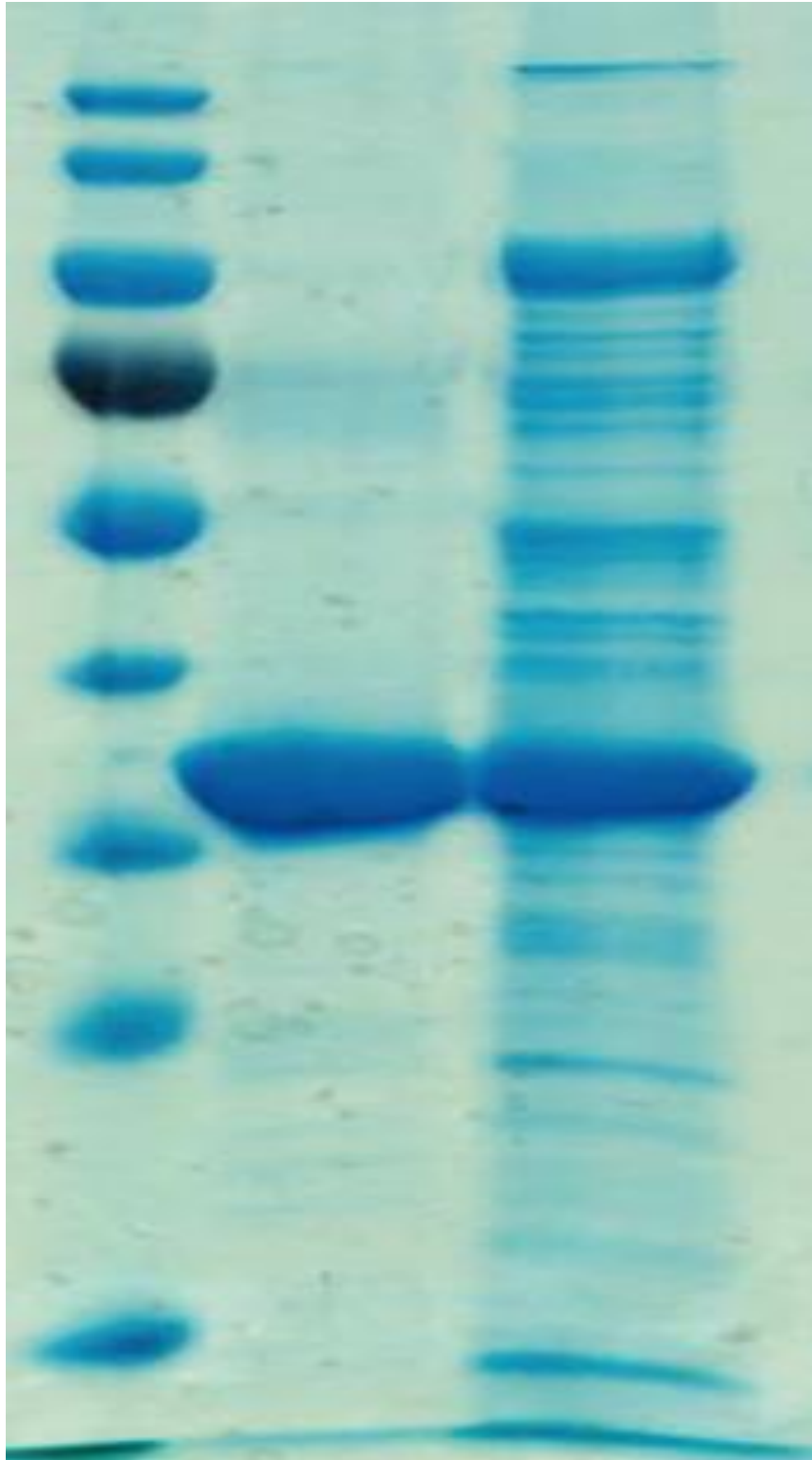


Figure A8 MMR results for Glt_{Tk} variants. Protomers are shown in an outward open (left, PDB 4KY0) and 2 inward open with 1 intermediate outward occluded (right, PDB 6XWR) with MTSL rotamers shown as red clusters. The transport domain (green) and scaffold domain (pink) are shown, with HP1 (blue) and HP2 (orange) highlighted.

A5 EasySpin spectral simulations of immobilised Glt_{TK} S281C

A5.1 EasySpin simulation results for Glt_{TK} S281C in detergent micelles at 50K

	g_{zz}	g_{yy}	g_{xx}	A_{xx} (MHz)	A_{yy} (MHz)	A_{zz} (MHz)	Lwpp (mT)
Apo	2.0078	2.0054	2.0015	16.450	11.269	102.794	0.80
Na⁺	2.0078	2.0054	2.0015	17.457	11.472	102.712	0.82
Substrate	2.0078	2.0054	2.0016	15.784	10.336	102.792	0.83
Inhibitor	2.0078	2.0055	2.0016	15.364	12.139	102.797	0.83

Table A2 Best fit simulation values for LT X-band cw-EPR spectra of Glt_{TK} S281C in detergent micelles. Results are shown for the four binding states with RMSD values of 0.6000 (apo), 0.5859 (Na⁺), 0.5003 (Na⁺ and L-aspartate), and 0.5396 (Na⁺ and DL-TBOA). The x, y and z components of the g- and A-tensor and the linewidth (peak to peak, lwpp) are shown for each.

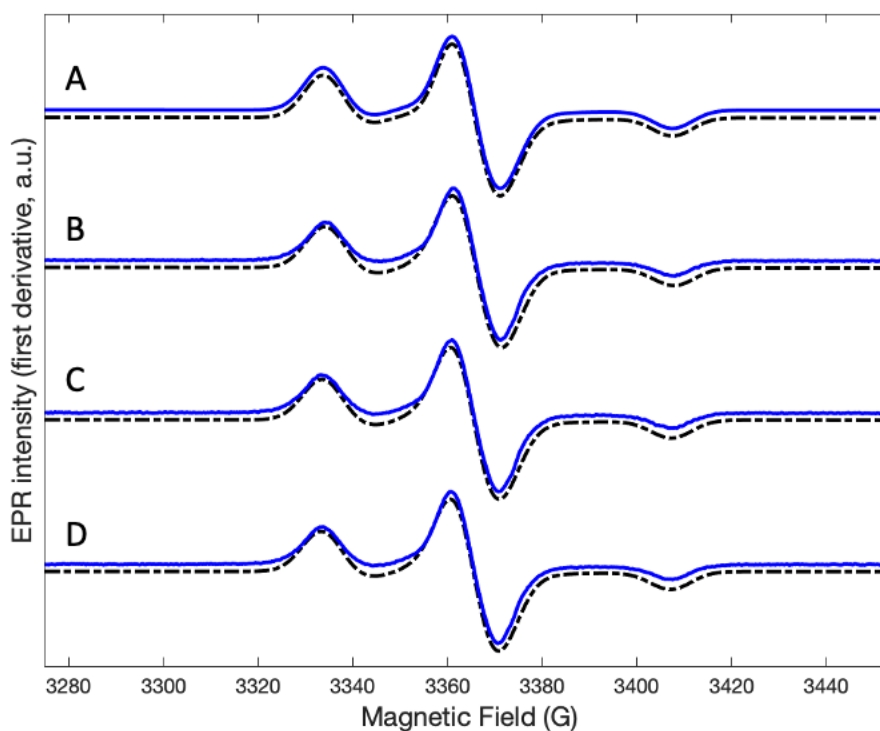


Figure A9 LT X-band cw-EPR spectra for Glt_{TK} S281C in detergent micelles (blue) and their respective simulations using EasySpin (black dashed line) for the proposed intermediates of the transport cycle: A) apo, B) Na⁺ (50 mM), C) substrate bound (50 mM Na⁺ and 1 mM L-aspartate) and D) inhibitor bound (50 mM Na⁺ and 1 mM DL-TBOA)

A5.2 EasySpin simulation results for cw Gl_{Tk} S281C in liposomes at 50K

	g_{zz}	g_{yy}	g_{xx}	A_{xx} (MHz)	A_{yy} (MHz)	A_{zz} (MHz)	Lwpp (mT)
Apo	2.0078	2.0054	2.0015	16.450	11.269	102.794	0.76
Na⁺	2.0078	2.0054	2.0015	17.457	11.472	102.712	0.79
Substrate	2.0078	2.0054	2.0016	15.784	10.336	102.792	0.79
Inhibitor	2.0078	2.0055	2.0016	15.364	12.139	102.797	0.77

Table A3 Best fit simulation values for LT X-band cw-EPR spectra of Gl_{Tk} S281C in liposomes. Results are shown for the four binding states with RMSD values of 0.6466 (apo), 0.9361 (Na⁺), 1.0843 (Na⁺ and L-aspartate), and 0.6708 (Na⁺ and DL-TBOA). The x, y and z components of the g- and A-tensor and the linewidth (peak to peak, lwpp) are shown for each.

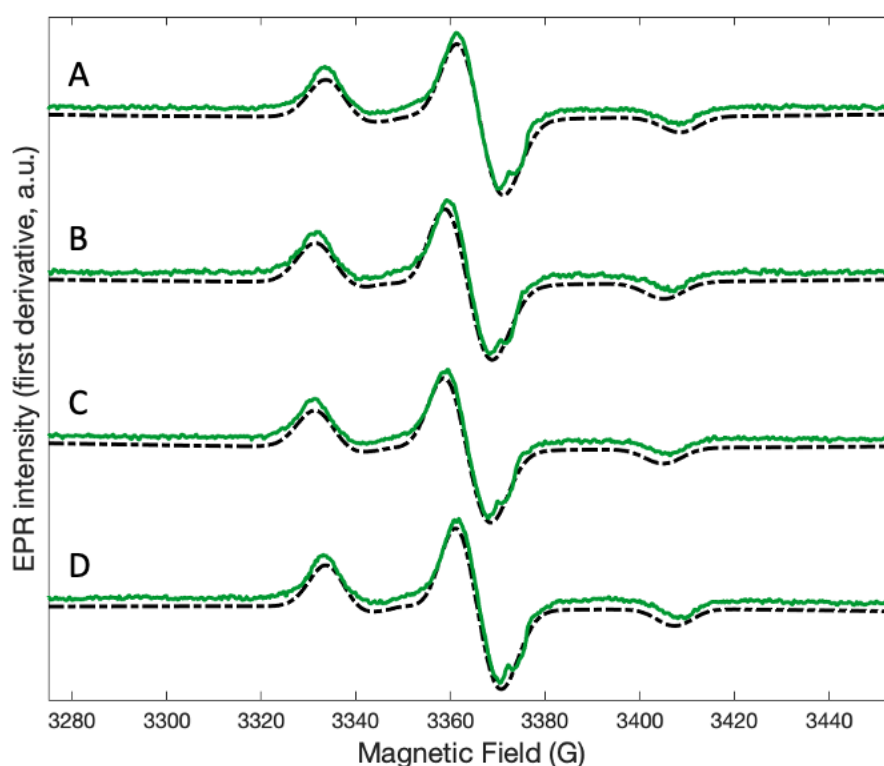


Figure A10 LT X-band cw-EPR spectra for Gl_{Tk} S281C in liposomes (green) and their respective simulations using EasySpin (black dashed line) for the proposed intermediates of the transport cycle: A) apo, B) Na⁺ (50 mM), C) substrate bound (50 mM Na⁺ and 1 mM L-aspartate) and D) inhibitor bound (50 mM Na⁺ and 1 mM DL-TBOA)

A5.3 EasySpin simulation results for Glt_{7k} S281C in SMALPs at 50K

	g_{zz}	g_{yy}	g_{xx}	A_{xx} (MHz)	A_{yy} (MHz)	A_{zz} (MHz)	Lwpp (mT)
Apo	2.0078	2.0054	2.0015	16.450	11.269	102.794	0.80
Na⁺	2.0078	2.0054	2.0015	17.457	11.472	102.712	0.80
Substrate	2.0078	2.0054	2.0016	15.784	10.336	102.792	0.81
Inhibitor	2.0078	2.0055	2.0016	15.364	12.139	102.797	0.80

Table A4 Best fit simulation values for LT X-band cw-EPR spectra of Glt_{7k} S281C in SMALPs. Results are shown for the four binding states with RMSD values of 1.2911 (apo), 1.3375 (Na⁺), 1.3673 (Na⁺ and L-aspartate), and 1.4872 (Na⁺ and DL-TBOA). The x, y and z components of the g- and A-tensor and the linewidth (peak to peak, lwpp) are shown for each.

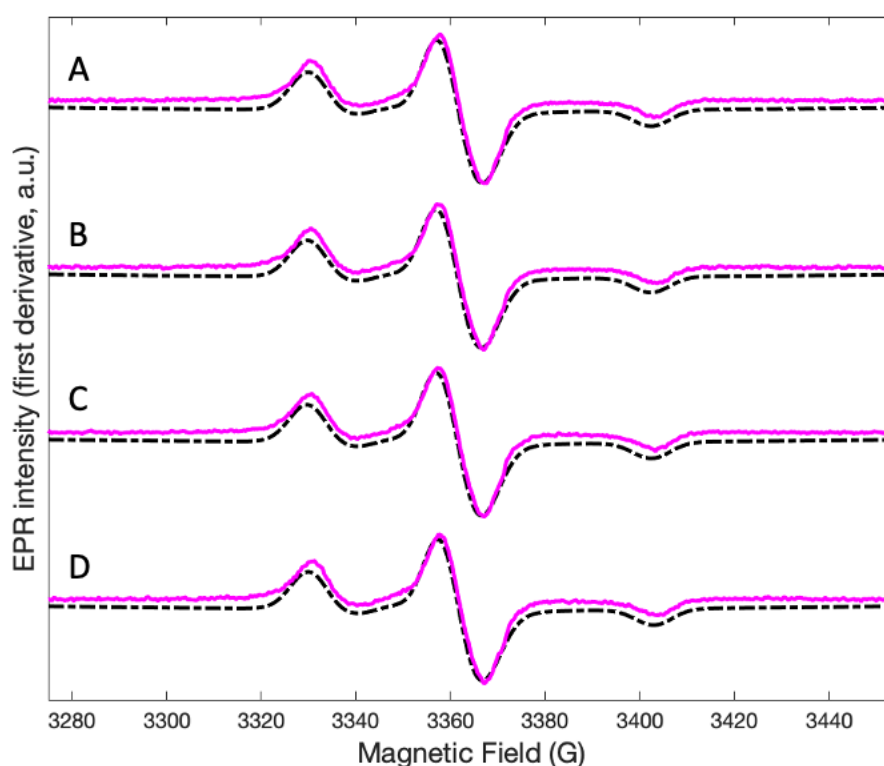


Figure A11 LT X-band cw-EPR spectra for Glt_{7k} S281C in SMALPs (pink) and their respective simulations using EasySpin (black dashed line) for the proposed intermediates of the transport cycle: A) apo, B) Na⁺ (50 mM), C) substrate bound (50 mM Na⁺ and 1 mM L-aspartate) and D) inhibitor bound (50 mM Na⁺ and 1 mM DL-TBOA)

A5.4 EasySpin simulation results for Glt_{TK} S281C in nanodiscs at 50K

	g_{zz}	g_{yy}	g_{xx}	A_{xx} (MHz)	A_{yy} (MHz)	A_{zz} (MHz)	Lwpp (mT)
Apo	2.0078	2.0054	2.0015	16.450	11.269	102.794	0.76
Na⁺	2.0078	2.0054	2.0015	17.457	11.472	102.712	0.74
Substrate	2.0078	2.0054	2.0016	15.784	10.336	102.792	0.76
Inhibitor	2.0078	2.0055	2.0016	15.364	12.139	102.797	0.75

Table A5 Best fit simulation values for LT X-band cw-EPR spectra of Glt_{TK} S281C in nanodiscs. Results are shown for the four binding states with RMSD values of 0.5319 (apo), 0.5844 (Na⁺), 0.9616 (Na⁺ and L-aspartate), and 0.8280 (Na⁺ and DL-TBOA). The x, y and z components of the g- and A-tensor and the linewidth (peak to peak, lwpp) are shown for each.

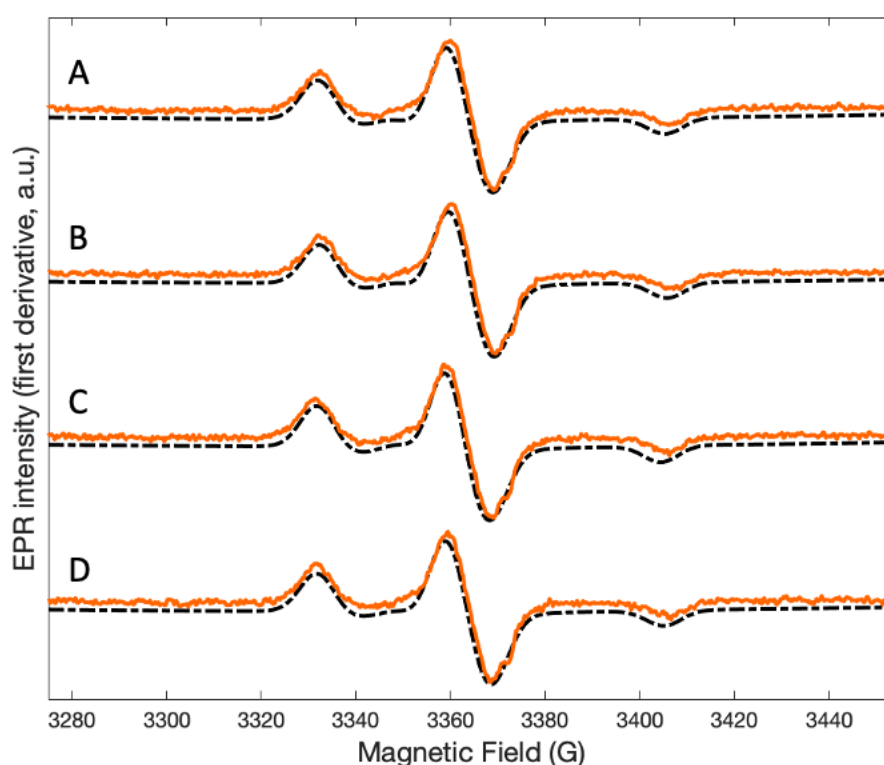


Figure A12 LT X-band cw-EPR spectra for Glt_{TK} S281C in nanodiscs (orange) and their respective simulations using EasySpin (black dashed line) for the proposed intermediates of the transport cycle: A) apo, B) Na⁺ (50 mM), C) substrate bound (50 mM Na⁺ and 1 mM L-aspartate) and D) inhibitor bound (50 mM Na⁺ and 1 mM DL-TBOA)

A6 EasySpin spectral simulations of immobilised Glt_{TK} D298C

A6.1 EasySpin simulation results for Glt_{TK} D298C in detergent micelles at 50K

	g_{zz}	g_{yy}	g_{xx}	A_{xx} (MHz)	A_{yy} (MHz)	A_{zz} (MHz)	Lwpp (mT)
Apo	2.0078	2.0057	2.0018	11.2	17.1	101.1	0.81
Na⁺	2.0078	2.0058	2.0017	15.2	17.4	101.1	0.82
Substrate	2.0078	2.0057	2.0017	15.6	16.1	101.1	0.82
Inhibitor	2.0078	2.0057	2.0018	16.6	15.9	101.1	0.82

Table A6 Best fit simulation values for LT X-band cw-EPR spectra of Glt_{TK} D298C in detergent micelles. Results are shown for the four binding states with RMSD values of 0.4225 (apo), 0.4087 (Na⁺), 0.5108 (Na⁺ and L-aspartate), and 0.4525 (Na⁺ and DL-TBOA). The x, y and z components of the g- and A-tensor and the linewidth (peak to peak, lwpp) are shown for each.

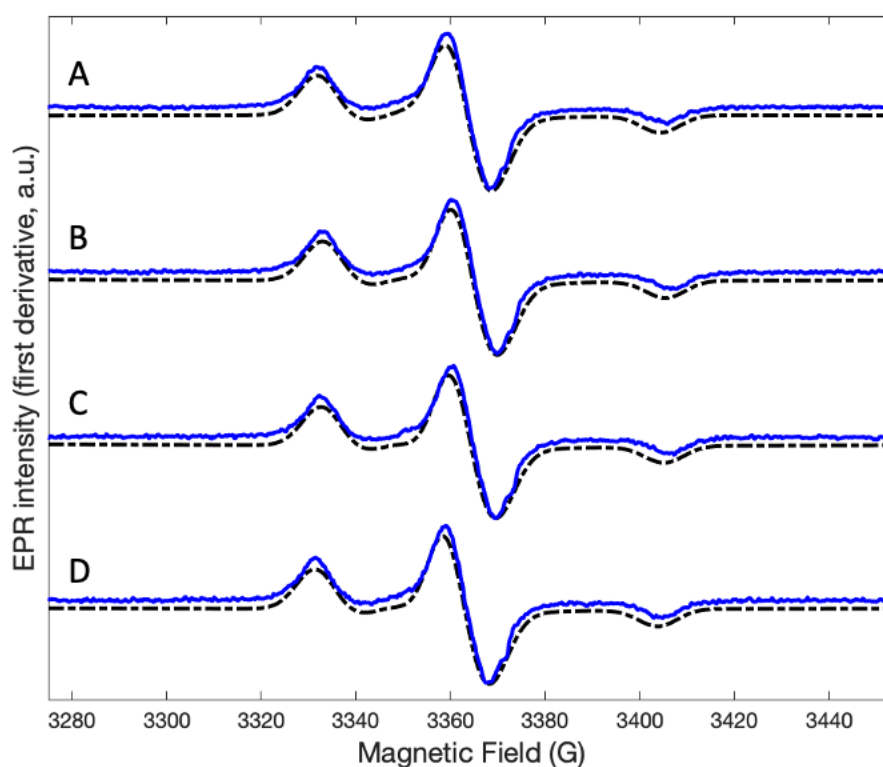


Figure A13 LT X-band cw-EPR spectra for Glt_{TK} D298C in detergent micelles (blue) and their respective simulations using EasySpin (black dashed line) for the proposed intermediates of the transport cycle: A) apo, B) Na⁺ (50 mM), C) substrate bound (50 mM Na⁺ and 1 mM L-aspartate) and D) inhibitor bound (50 mM Na⁺ and 1 mM DL-TBOA)

A6.2 EasySpin simulation results for Glt_{7k} D298C in liposomes at 50K

	g_{zz}	g_{yy}	g_{xx}	A_{xx} (MHz)	A_{yy} (MHz)	A_{zz} (MHz)	Lwpp (mT)
Apo	2.0078	2.0057	2.0018	11.2	17.1	101.1	0.80
Na⁺	2.0078	2.0058	2.0017	15.2	17.4	101.1	0.81
Substrate	2.0078	2.0057	2.0017	15.6	16.1	101.1	0.81
Inhibitor	2.0078	2.0057	2.0018	16.6	15.9	101.1	0.81

Table A7 Best fit simulation values for LT X-band cw-EPR spectra of Glt_{7k} D298C in liposomes. Results are shown for the four binding states with RMSD values of 1.2383 (apo), 1.0992 (Na⁺), 1.4977 (Na⁺ and L-aspartate), and 1.0727 (Na⁺ and DL-TBOA). The x, y and x components of the g- and A-tensor and the linewidth (peak to peak, lwpp) are shown for each.

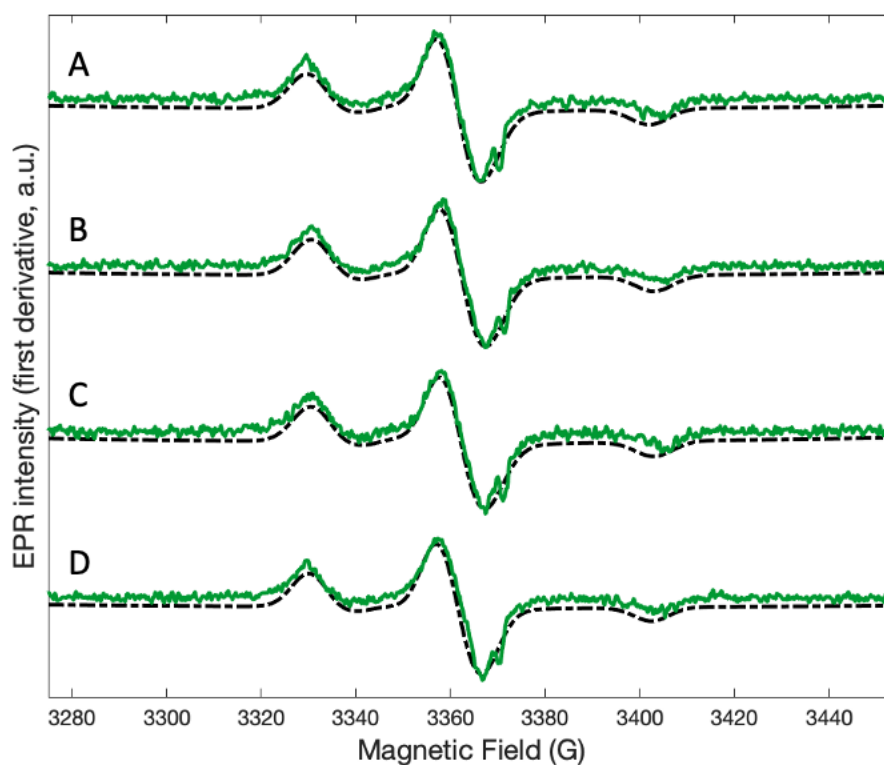


Figure A14 LT X-band cw-EPR spectra for Glt_{7k} D298C in liposomes (green) and their respective simulations using EasySpin (black dashed line) for the proposed intermediates of the transport cycle: A) apo, B) Na⁺ (50 mM), C) substrate bound (50 mM Na⁺ and 1 mM L-aspartate) and D) inhibitor bound (50 mM Na⁺ and 1 mM DL-TBOA)

A7 EasySpin spectral simulations of immobilised Glt_{TK} I312C

A7.1 EasySpin simulation results for Glt_{TK} I312C in detergent micelles at 50K

	g_{zz}	g_{yy}	g_{xx}	A_{xx} (MHz)	A_{yy} (MHz)	A_{zz} (MHz)	Lwpp (mT)
Apo	2.0081	2.0054	2.0015	15.3	12.1	92.9	0.69
Na⁺	2.0081	2.0055	2.0015	15.7	13.0	92.9	0.70
Substrate	2.0081	2.0054	2.0014	14.3	12.3	92.9	0.69
Inhibitor	2.0081	2.0056	2.0015	15.2	14.4	92.9	0.69

Table A8 Best fit simulation values for LT X-band cw-EPR spectra of Glt_{TK} I312C in detergent micelles. Results are shown for the four binding states with RMSD values of 0.3967 (apo), 0.3714 (Na⁺), 0.3167 (Na⁺ and L-aspartate), and 0.4107 (Na⁺ and DL-TBOA). The x, y and z components of the g- and A-tensor and the linewidth (peak to peak, lwpp) are shown for each.

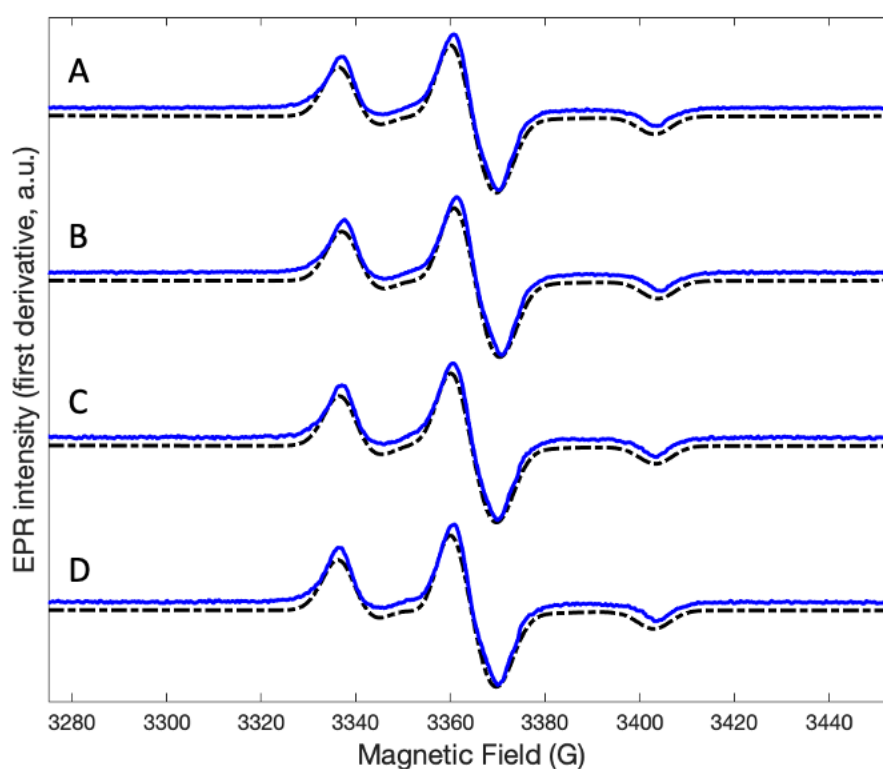


Figure A15 LT X-band cw-EPR spectra for Glt_{TK} I312C in detergent micelles (blue) and their respective simulations using EasySpin (black dashed line) for the proposed intermediates of the transport cycle: A) apo, B) Na⁺ (50 mM), C) substrate bound (50 mM Na⁺ and 1 mM L-aspartate) and D) inhibitor bound (50 mM Na⁺ and 1 mM DL-TBOA)

A7.2 EasySpin simulation results for Glt_{TK} I312C in liposomes at 50K

	g_{zz}	g_{yy}	g_{xx}	A_{xx} (MHz)	A_{yy} (MHz)	A_{zz} (MHz)	Lwpp (mT)
Apo	2.0081	2.0054	2.0015	15.3	12.1	92.9	0.73
Na⁺	2.0081	2.0055	2.0015	15.7	13.0	92.9	0.74
Substrate	2.0081	2.0054	2.0014	14.3	12.3	92.9	0.75
Inhibitor	2.0081	2.0056	2.0015	15.2	14.4	92.9	0.72

Table A9 Best fit simulation values for LT X-band cw-EPR spectra of Glt_{TK} I312C in liposomes. Results are shown for the four binding states with RMSD values of 0.6962 (apo), 0.7294 (Na⁺), 0.5292 (Na⁺ and L-aspartate), and 0.7233 (Na⁺ and DL-TBOA). The x, y and x components of the g- and A-tensor and the linewidth (peak to peak, lwpp) are shown for each.

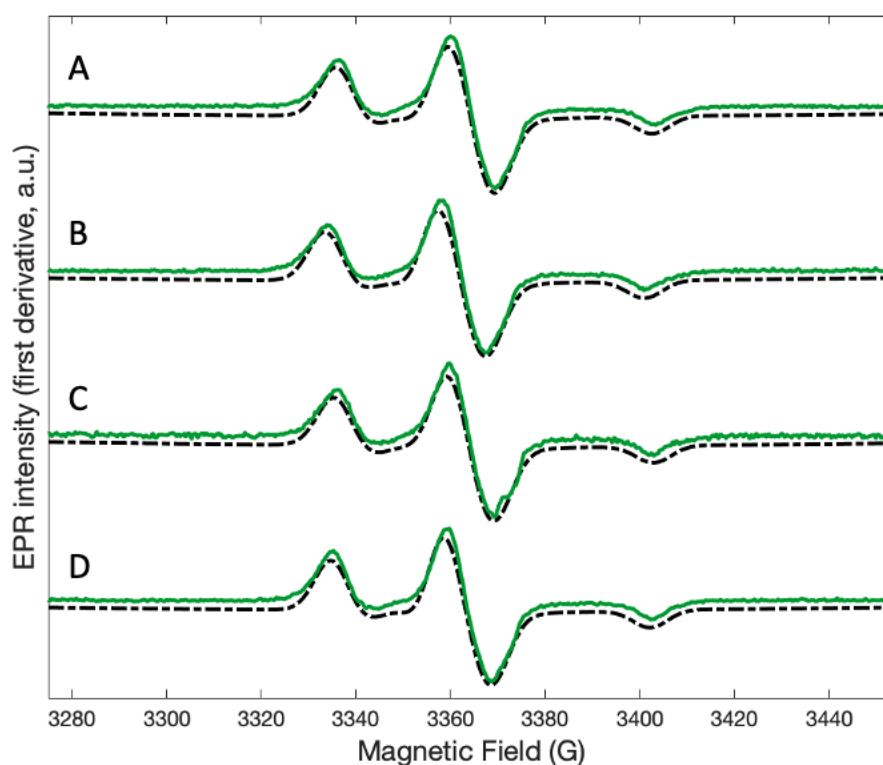


Figure A16 LT X-band cw-EPR spectra for Glt_{TK} I312C in liposomes (green) and their respective simulations using EasySpin (black dashed line) for the proposed intermediates of the transport cycle: A) apo, B) Na⁺ (50 mM), C) substrate bound (50 mM Na⁺ and 1 mM L-aspartate) and D) inhibitor bound (50 mM Na⁺ and 1 mM DL-TBOA)

A8 Functional studies of Glt_{Tk} M314C in detergent micelles and proteoliposomes using cw-EPR

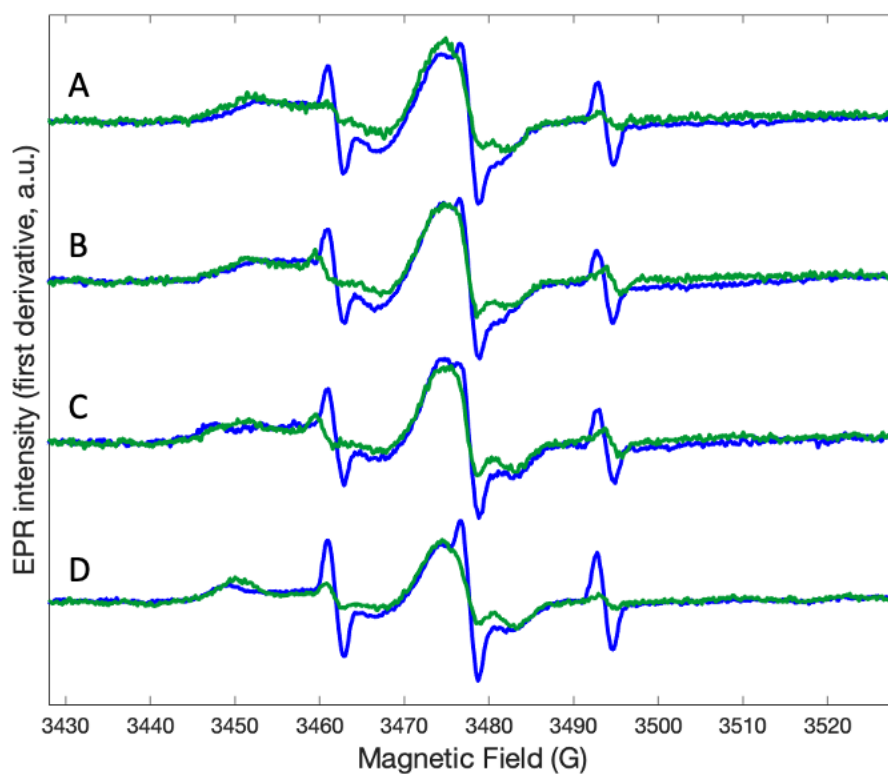


Figure A17 X-band RT cw-EPR spectra of Glt_{Tk} M314C in detergent micelles (blue) and liposomes (green) utilising substrate and co-transported Na⁺ to trap Glt_{Tk} in proposed intermediates of the transport cycle: A) apo, B) Na⁺ (50 mM), C) substrate bound (50 mM Na⁺ and 1 mM L-aspartate) and D) inhibitor bound (50 mM Na⁺ and 1 mM DL-TBOA). Experimental parameters: microwave frequency = 9.758 GHz, centre field = 3478 G, sweep width = 120 G, modulation frequency = 100 kHz, modulation amplitude = 1–2 G, microwave power = 0.1997 mW, number averages = 64–100

A9 EasySpin spectral simulations of immobilised Glt_{7k} M314C

A9.1 EasySpin simulation results for Glt_{7k} M314C in detergent micelles at 50K

	g_{zz}	g_{yy}	g_{xx}	A_{xx} (MHz)	A_{yy} (MHz)	A_{zz} (MHz)	Lwpp (mT)
Apo	2.0078	2.0058	2.0016	13.6	15.5	96.1	0.78
Na⁺	2.0078	2.0059	2.0017	13.3	16.5	96.1	0.79
Substrate	2.0078	2.0058	2.0016	14.8	15.5	96.1	0.81
Inhibitor	2.0078	2.0059	2.0017	14.5	15.8	96.1	0.84

Table A10 Best fit simulation values for LT X-band cw-EPR spectra of Glt_{7k} M314C in detergent micelles. Results are shown for the four binding states with RMSD values of 0.3710 (apo), 0.3935 (Na⁺), 0.5118 (Na⁺ and L-aspartate), and 0.6382 (Na⁺ and DL-TBOA). The x, y and z components of the g- and A-tensor and the linewidth (peak to peak, lwpp) are shown for each.

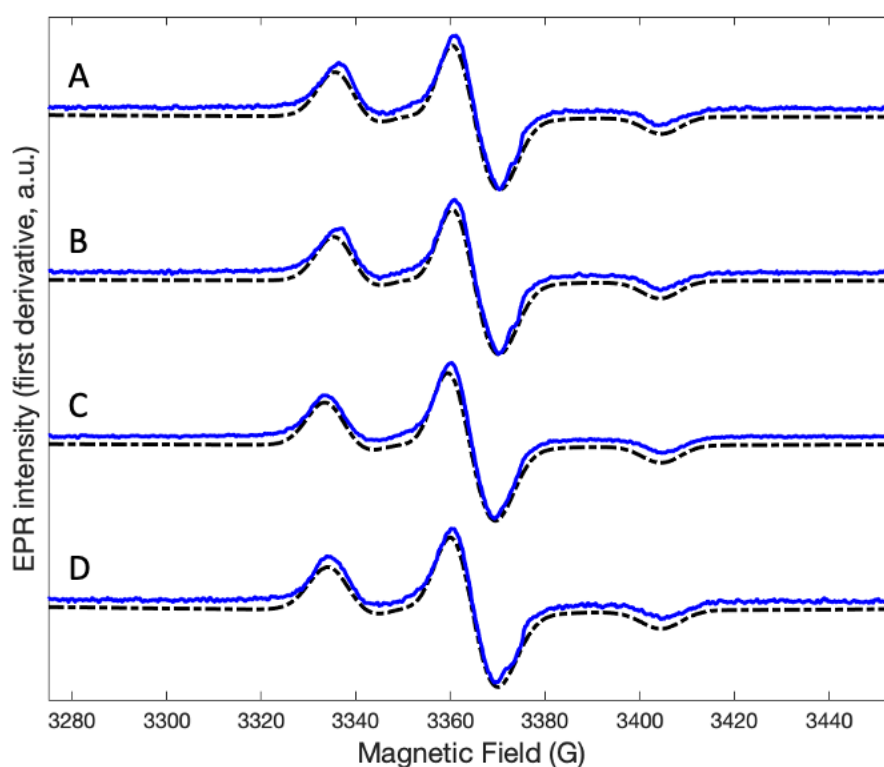


Figure A18 LT X-band cw-EPR spectra for Glt_{7k} M314C in detergent micelles (blue) and their respective simulations using EasySpin (black dashed line) for the proposed intermediates of the transport cycle: A) apo, B) Na⁺ (50 mM), C) substrate bound (50 mM Na⁺ and 1 mM L-aspartate) and D) inhibitor bound (50 mM Na⁺ and 1 mM DL-TBOA)

A9.2 EasySpin simulation results for Glt_{TK} M314C in liposomes at 50K

	g_{zz}	g_{yy}	g_{xx}	A_{xx} (MHz)	A_{yy} (MHz)	A_{zz} (MHz)	Lwpp (mT)
Apo	2.0077	2.0059	2.0015	14.5	11.8	96.1	0.76
Na⁺	2.0077	2.0057	2.0015	15.3	11.1	96.1	0.78
Substrate	2.0077	2.0059	2.0015	15.7	11.3	96.1	0.79
Inhibitor	2.0077	2.0057	2.0015	15.4	12.6	96.1	0.77

Table A11 Best fit simulation values for LT X-band cw-EPR spectra of Glt_{TK} M314C in liposomes. Results are shown for the four binding states with RMSD values of 0.6002 (apo), 0.5191 (Na⁺), 0.5956 (Na⁺ and L-aspartate), and 0.7174 (Na⁺ and DL-TBOA). The x, y and x components of the g- and A-tensor and the linewidth (peak to peak, lwpp) are shown for each.

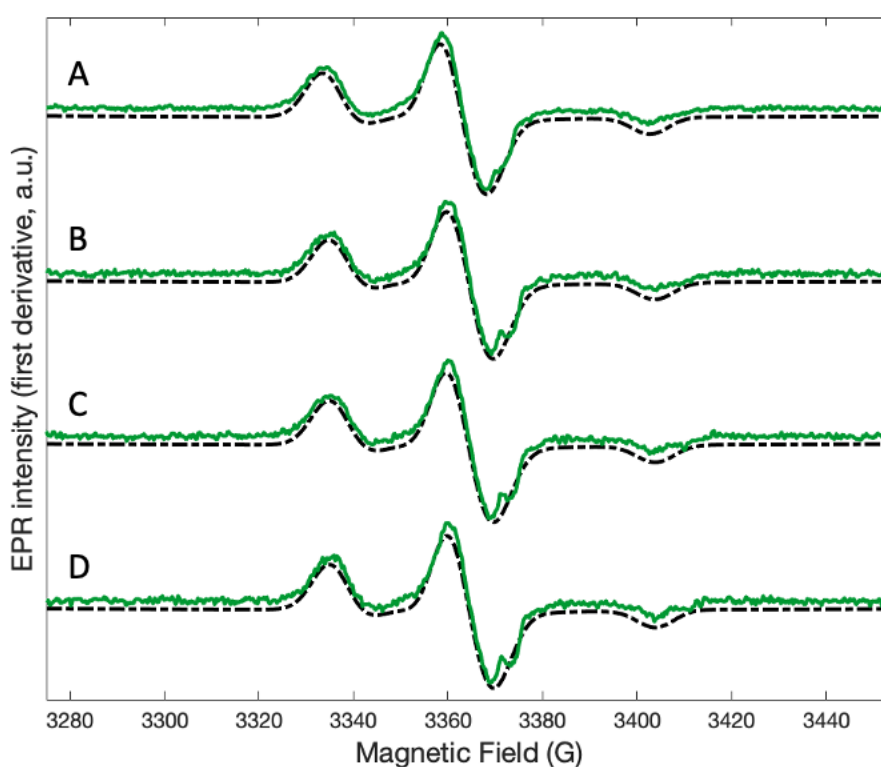


Figure A19 LT X-band cw-EPR spectra for Glt_{TK} M314C in liposomes (green) and their respective simulations using EasySpin (black dashed line) for the proposed intermediates of the transport cycle: A) apo, B) Na⁺ (50 mM), C) substrate bound (50 mM Na⁺ and 1 mM L-aspartate) and D) inhibitor bound (50 mM Na⁺ and 1 mM DL-TBOA)

A10 EasySpin spectral simulations of immobilised Glt_{TK} H334C

A10.1 EasySpin simulation results for Glt_{TK} H334C in detergent micelles at 50K

	g_{zz}	g_{yy}	g_{xx}	A_{xx} (MHz)	A_{yy} (MHz)	A_{zz} (MHz)	Lwpp (mT)
Apo	2.0078	2.0052	2.0016	15.5	14.5	101.4	0.73
Na⁺	2.0078	2.0052	2.0017	14.7	13.9	101.4	0.72
Substrate	2.0078	2.0053	2.0017	14.2	13.2	101.4	0.71
Inhibitor	2.0078	2.0052	2.0016	14.8	14.1	101.4	0.73

Table A12 Best fit simulation values for LT X-band cw-EPR spectra of Glt_{TK} H334C in detergent micelles. Results are shown for the four binding states with RMSD values of 0.4989 (apo), 0.4217 (Na⁺), 0.4704 (Na⁺ and L-aspartate), and 0.6101 (Na⁺ and DL-TBOA). The x, y and z components of the g- and A-tensor and the linewidth (peak to peak, lwpp) are shown for each.

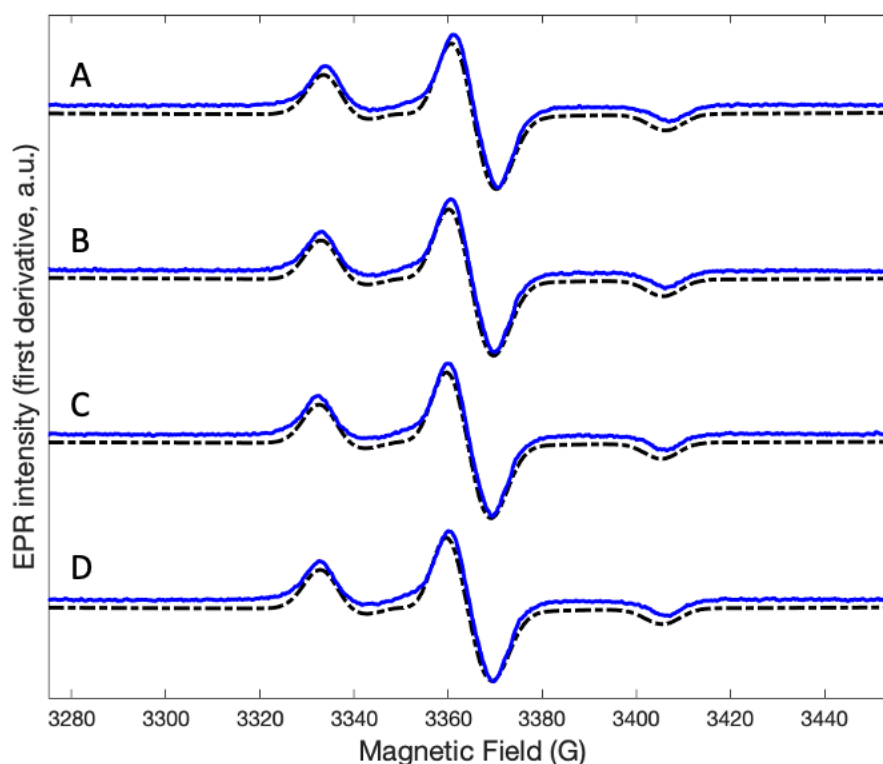


Figure A20 LT X-band cw-EPR spectra for Glt_{TK} H334C in detergent micelles (blue) and their respective simulations using EasySpin (black dashed line) for the proposed intermediates of the transport cycle: A) apo, B) Na⁺ (50 mM), C) substrate bound (50 mM Na⁺ and 1 mM L-aspartate) and D) inhibitor bound (50 mM Na⁺ and 1 mM DL-TBOA)

A10.2 EasySpin simulation results for Glt_{7k} H334C in liposomes at 50K

	g_{zz}	g_{yy}	g_{xx}	A_{xx} (MHz)	A_{yy} (MHz)	A_{zz} (MHz)	Lwpp (mT)
Apo	2.0078	2.0052	2.0016	15.5	14.5	101.4	0.73
Na⁺	2.0078	2.0052	2.0017	14.7	13.9	101.4	0.72
Substrate	2.0078	2.0053	2.0017	14.2	13.2	101.4	0.70
Inhibitor	2.0078	2.0052	2.0016	14.8	14.1	101.4	0.70

Table A13 Best fit simulation values for LT X-band cw-EPR spectra of Glt_{7k} H334C in liposomes. Results are shown for the four binding states with RMSD values of 0.7018 (apo), 0.7854 (Na⁺), 0.7550 (Na⁺ and L-aspartate), and 0.9770 (Na⁺ and DL-TBOA). The x, y and z components of the g- and A-tensor and the linewidth (peak to peak, lwpp) are shown for each.

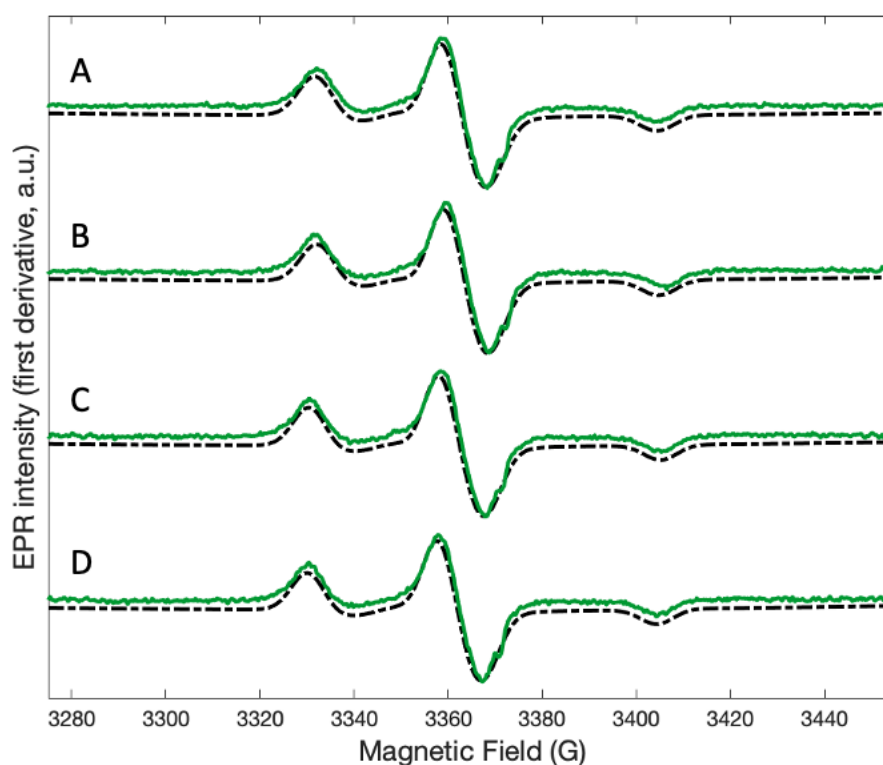


Figure A21 LT X-band cw-EPR spectra for Glt_{7k} H334C in liposomes (green) and their respective simulations using EasySpin (black dashed line) for the proposed intermediates of the transport cycle: A) apo, B) Na⁺ (50 mM), C) substrate bound (50 mM Na⁺ and 1 mM L-aspartate) and D) inhibitor bound (50 mM Na⁺ and 1 mM DL-TBOA)

A11 EasySpin spectral simulations of immobilised Glt_{TK} A367C

A11.1 EasySpin simulation results for Glt_{TK} A367C in detergent micelles at 50K

	g_{zz}	g_{yy}	g_{xx}	A_{xx} (MHz)	A_{yy} (MHz)	A_{zz} (MHz)	Lwpp (mT)
Apo	2.0076	2.0054	2.0015	13.3	15.1	101.4	0.80
Na⁺	2.0076	2.0054	2.0015	15.2	14.7	101.4	0.79
Substrate	2.0076	2.0055	2.0014	13.6	15.7	101.4	0.79
Inhibitor	2.0076	2.0053	2.0015	12.8	15.6	101.4	0.77

Table A14 Best fit simulation values for LT X-band cw-EPR spectra of Glt_{TK} A367C in detergent micelles. Results are shown for the four binding states with RMSD values of 0.3080 (apo), 0.4147 (Na⁺), 0.3807 (Na⁺ and L-aspartate), and 0.3350 (Na⁺ and DL-TBOA). The x, y and z components of the g- and A-tensor and the linewidth (peak to peak, lwpp) are shown for each.

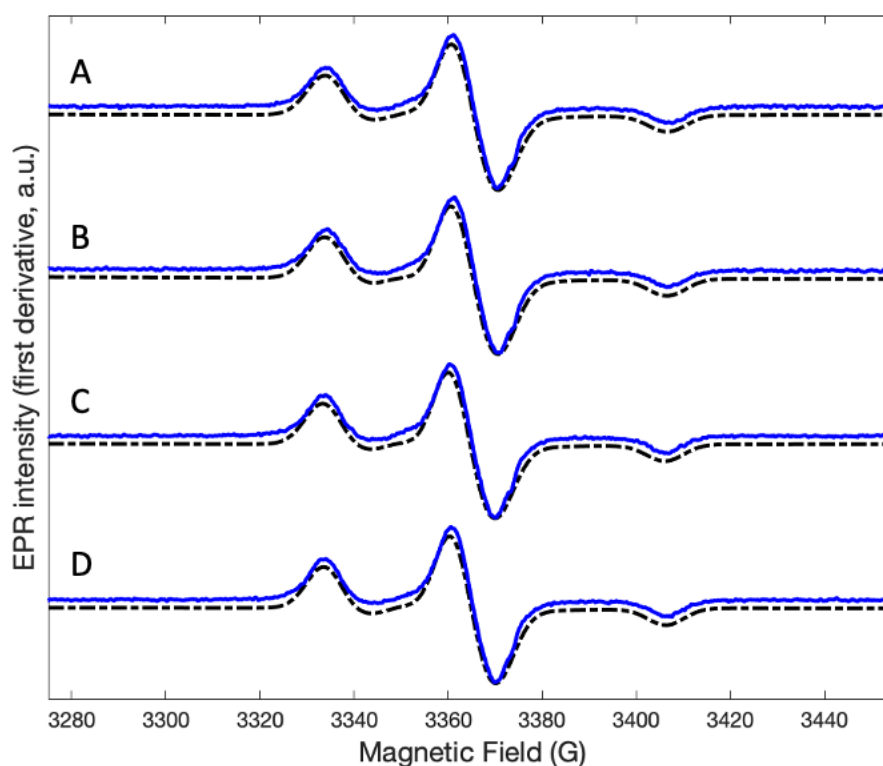


Figure A22 LT X-band cw-EPR spectra for Glt_{TK} A367C in detergent micelles (blue) and their respective simulations using EasySpin (black dashed line) for the proposed intermediates of the transport cycle: A) apo, B) Na⁺ (50 mM), C) substrate bound (50 mM Na⁺ and 1 mM L-aspartate) and D) inhibitor bound (50 mM Na⁺ and 1 mM DL-TBOA)

A11.2 EasySpin simulation results for Glt_{Tk} A367C in liposomes at 50K

	g_{zz}	g_{yy}	g_{xx}	A_{xx} (MHz)	A_{yy} (MHz)	A_{zz} (MHz)	Lwpp (mT)
Apo	2.0076	2.0054	2.0015	13.3	15.1	101.4	0.81
Na⁺	2.0076	2.0054	2.0015	15.2	14.7	101.4	0.81
Substrate	2.0076	2.0055	2.0014	13.6	15.7	101.4	0.83
Inhibitor	2.0076	2.0053	2.0015	12.8	15.6	101.4	0.78

Table A15 Best fit simulation values for LT X-band cw-EPR spectra of Glt_{Tk} A367C in liposomes. Results are shown for the four binding states with RMSD values of 0.4434 (apo), 0.4069 (Na⁺), 0.6338 (Na⁺ and L-aspartate), and 0.5131 (Na⁺ and DL-TBOA). The x, y and z components of the g- and A-tensor and the linewidth (peak to peak, lwpp) are shown for each.

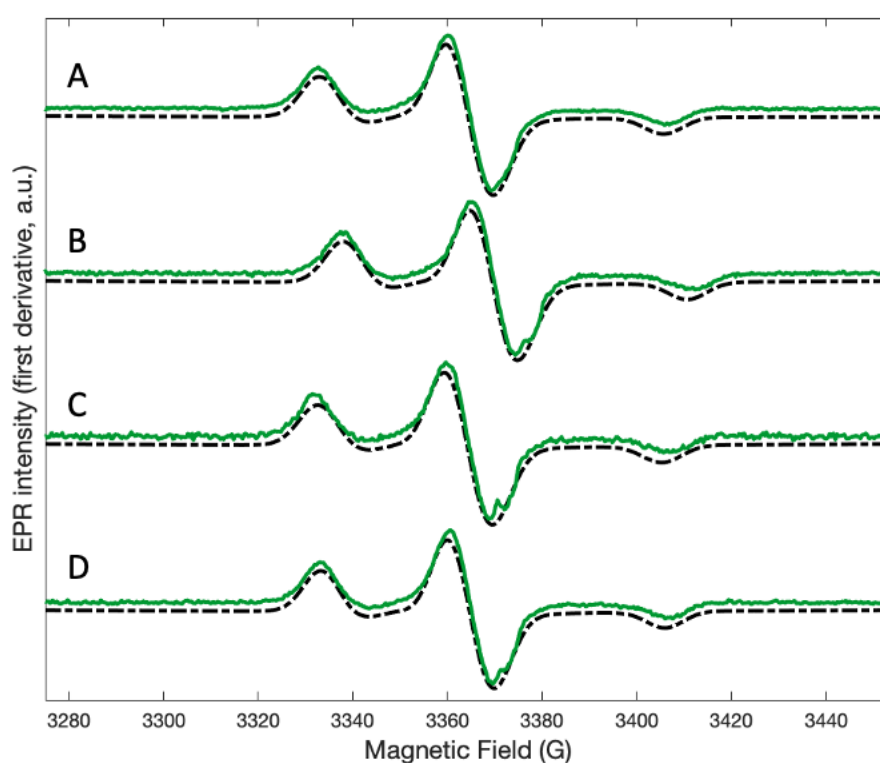


Figure A23 LT X-band cw-EPR spectra for Glt_{Tk} A367C in liposomes (green) and their respective simulations using EasySpin (black dashed line) for the proposed intermediates of the transport cycle: A) apo, B) Na⁺ (50 mM), C) substrate bound (50 mM Na⁺ and 1 mM L-aspartate) and D) inhibitor bound (50 mM Na⁺ and 1 mM DL-TBOA)

A11.3 EasySpin simulation results for Glt_{TK} A367C in SMALPs at 50K

	g_{zz}	g_{yy}	g_{xx}	A_{xx} (MHz)	A_{yy} (MHz)	A_{zz} (MHz)	Lwpp (mT)
Apo	2.0076	2.0054	2.0015	14.6	14.2	101.4	0.72
Na⁺	2.0076	2.0054	2.0015	13.3	13.6	101.4	0.76
Substrate	2.0076	2.0055	2.0015	14.2	14.5	101.4	0.74
Inhibitor	2.0076	2.0054	2.0015	14.1	13.8	101.4	0.74

Table A16 Best fit simulation values for LT X-band cw-EPR spectra of Glt_{TK} A367C in SMALPs. Results are shown for the four binding states with RMSD values of 0.6421 (apo), 0.5523 (Na⁺), 0.6155 (Na⁺ and L-aspartate), and 0.6276 (Na⁺ and DL-TBOA). The x, y and x components of the g- and A-tensor and the linewidth (peak to peak, lwpp) are shown for each.

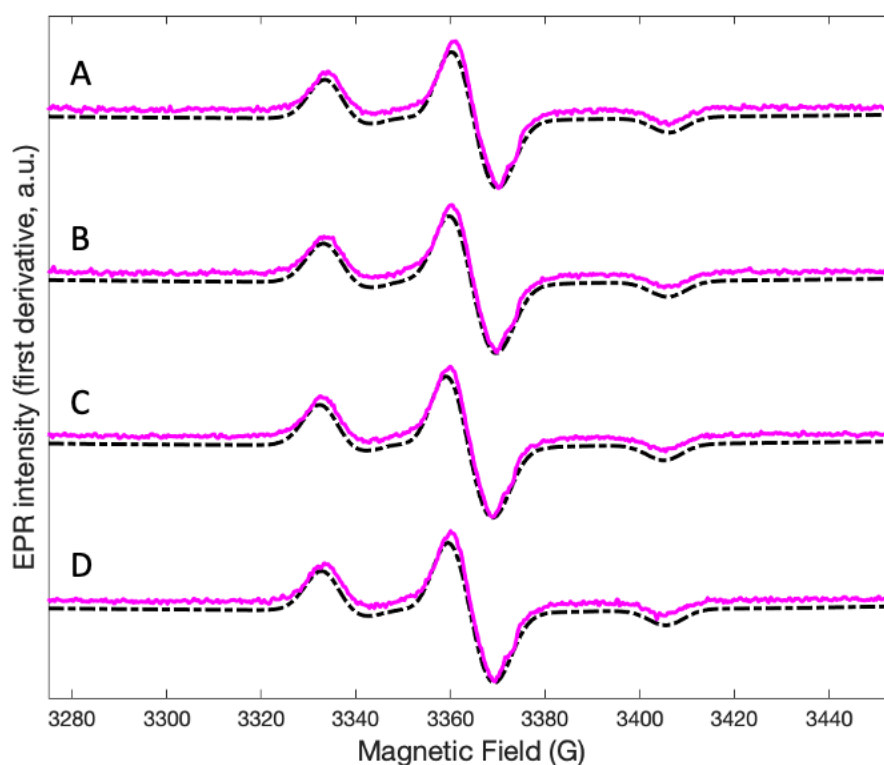


Figure A24 LT X-band cw-EPR spectra for Glt_{TK} A367C in SMALPs (pink) and their respective simulations using EasySpin (black dashed line) for the proposed intermediates of the transport cycle: A) apo, B) Na⁺ (50 mM), C) substrate bound (50 mM Na⁺ and 1 mM L-aspartate) and D) inhibitor bound (50 mM Na⁺ and 1 mM DL-TBOA)

A12 Addition of extra L-aspartate to Glt_{7k} S281C in SMALPs

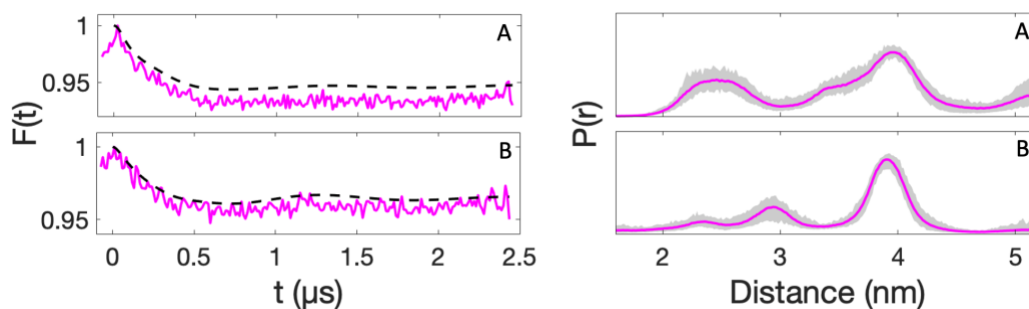


Figure A25 4-pulse DEER experiments (50 K) for substrate-bound Glt_{7k} S281C in SMALPs with the addition of excess L-aspartate. A) Glt_{7k} S281C substrate bound (50 mM Na⁺ and 1 mM L-aspartate, top) and B) following the addition of excess substrate (50 mM L-aspartate). The normalised background corrected time decays are shown on the left with their respective fittings shown as black dashed lines. The right hand side spectra shown the calculated distance distributions using DeerNet with 95% confidence intervals represented by the light grey areas.

A13 The effect of adding d₈-glycerol on the T₂ spectrum of Glt_{7k} M314C

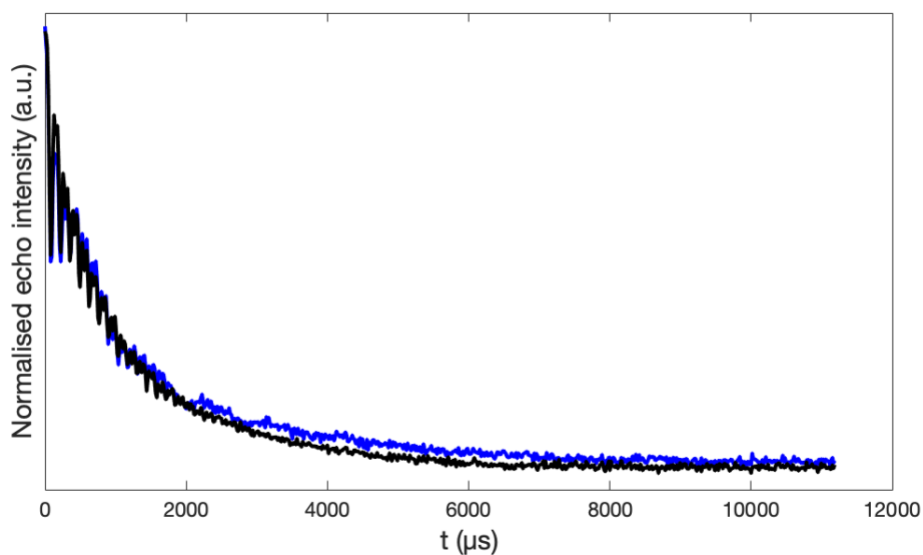


Figure A26 The effects of glycerol on the T₂ experiment of Glt_{7k} M314C in an apo state. The spectra shown represent no glycerol present (black) and after the addition of 30% v/v d₈-glycerol (blue) where oscillations would typically appear from the presence of deuterium.

A14 Glt_{TK} M314C pulse EPR spectroscopy for distance determination in detergent micelles and liposomes

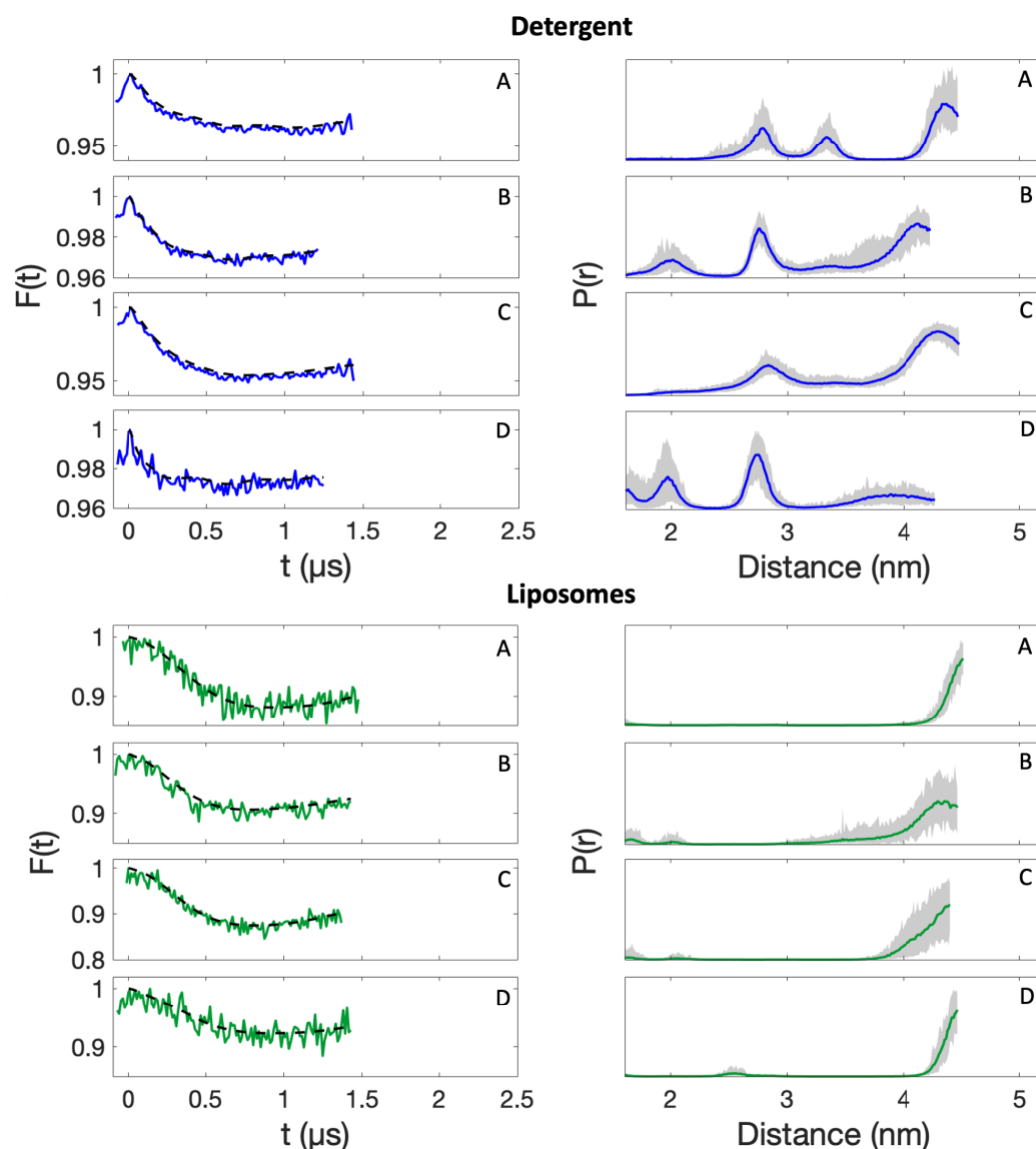


Figure A27 4-pulse DEER experiments (50 K) for Glt_{TK} M314C in detergent micelles (blue) and liposomes (green) utilising substrate and co-transported Na⁺ to trap Glt_{TK} in proposed intermediates of the transport cycle: A) apo, B) Na⁺ (50 mM), C) substrate bound (50 mM Na⁺ and 1 mM L-aspartate) and D) inhibitor bound (50 mM Na⁺ and 1 mM DL-TBOA). The normalised background corrected time decays are shown on the left with their respective fittings shown as black dashed lines. The right hand side spectra shown the calculated distance distributions using DeerNet with 95% confidence intervals represented by the light grey areas.

A15 Inversion recovery filtered DEER (iDEER)

The filter time, T_F , describes the time where each species traverses the zero-crossing point of magnetisation M_z . Here the Hahn echo is not observed and the species would be suppressed. T_F is used to filter the contribution of the echo for one species, in a sample containing two-species i.e. Cu^{2+} -NTA and MTSL, meaning only the other is detected. Cu^{2+} species require smaller T_F compared to MTSL which is the basis of the selective filtering in iDEER. The iDEER pulse sequence closely resembles that of the 4-pulse DEER experiment, with the addition of an initial inversion pulse in the observe frequency, ω_A . This perturbs A-spins but does not affect B-spins in the pump frequency, ω_B . After the inversion pulse, the spins relax via T_1 magnetisation where at a specific time, T_F , the A-spins pass through zero. These have been suppressed as no Hahn echo is observed in the detection sequence. For example, in orthogonally labelled sample both the Cu^{2+} and MTSL will have A-spins (in the observe frequency) and B-spins (in the pump frequency). If a T_F is selected for the Cu^{2+} species then the echo of the corresponding A-spins is suppressed, but the B-spins are still excited in the pump frequency. Meanwhile the MTSL A- and B-spins are excited in the respective frequencies as in a normal 4-pulse DEER experiment. This filters out the dipolar interaction between the A- and B-spins of the same Cu^{2+} species, with all other interactions observed.

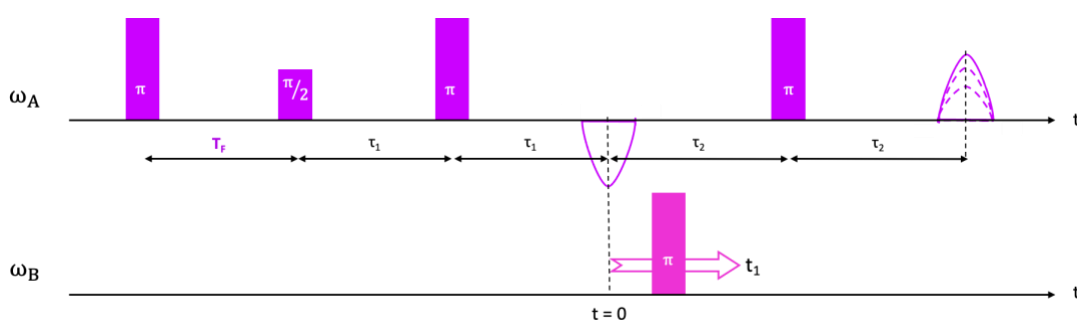


Figure A28 Pulse sequence for the iDEER experiment following the initial inversion π pulse, with filter time labelled T_F . Two different frequencies are used: the observe frequency, ω_A , and the pump frequency, ω_B .

A16 *In silico* modelling of Glt_{ph} using MMM

For Glt_{ph} variants the following names correspond to the following mutations. CM3: C321S G280C F50H L54H; CM7: C321S G149C V51H K55H; CM8: C321S T375C V51H K55H; CM10: C321S G351C K132H D136H; CM11: C321S R52C A364H E368H

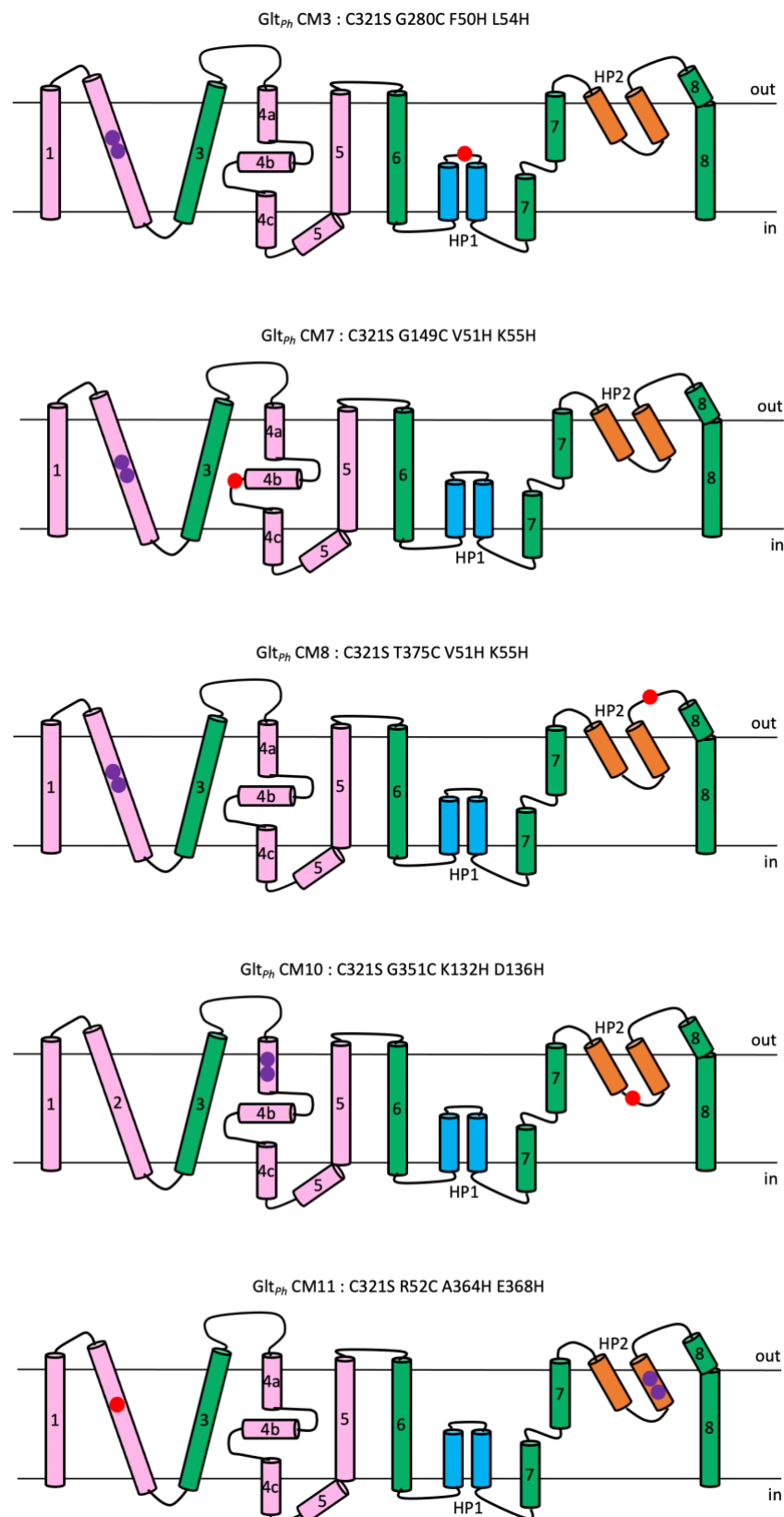


Figure A29 Glt_{ph} membrane topology highlighting the locations of the cysteine residues and dHis motif for each variant. MTSL-binding sites are shown as red dots to represent cysteine residues, and the dHis motif as purple dots for Cu²⁺-NTA binding. It is composed of a transport domain (green) and scaffold domain (pink), with HP1 (blue) and HP2 (orange) highlighted.

State	Conformation(s)	Variant	Rotamers	Distances (nm)
Apo ^I	Outward facing occluded	CM3	10 / 11 / 10	3.23 / 3.30 / 3.35
		CM8	135 / 136 / 133	3.66 / 3.67 / 3.70
		CM10	35 / 34 / 34	4.14 / 4.13 / 4.16
		CM11	11 / 13 / 12	2.91 / 3.10 / 3.04
Apo ^{II}	Inward facing occluded	CM3	61 / 62 / 62	4.52 / 4.52 / 4.49
		CM8	146 / 142 / 144	2.61 / 2.62 / 2.67
		CM10	5 / 6 / 4	2.97 / 2.82 / 2.95
		CM11	17 / 17 / 18	2.93 / 2.80 / 2.92
Na ⁺ only ^{III}	Outward occluded	CM3	5 / 5 / 5	3.02 / 3.32 / 3.52
		CM8	149 / 149 / 149	3.85 / 3.83 / 3.87
		CM10	60 / 59 / 60	4.33 / 4.45 / 4.37
		CM11	13 / 14 / 12	3.04 / 2.97 / 2.98
Substrate bound ^{IV}	Inward facing occluded	CM3	84 / 86 / 85	4.12 / 4.13 / 4.11
		CM8	118 / 118 / 114	2.20 / 2.26 / 2.22
		CM10	1 / 1 / 1	3.52 / 3.52 / 3.53
		CM11	28 / 29 / 27	2.96 / 2.98 / 2.97
Substrate bound ^V	Inward facing	CM3	85 / 85 / 85	4.54 / 4.54 / 4.54
		CM8	117 / 114 / 115	2.21 / 2.19 / 2.20
		CM10	1 / 1 / 1	3.15 / 3.15 / 3.15
		CM11	21 / 20 / 20	3.11 / 3.09 / 3.11
Inhibitor bound ^{VI}	Inward facing	CM3	57 / 57 / 57	4.54 / 4.56 / 4.56
		CM8	119 / 119 / 118	1.89 / 1.89 / 1.92
		CM10	1 / 1 / 1	2.60 / 2.59 / 2.61
		CM11	18 / 15 / 18	2.97 / 2.98 / 2.99
Inhibitor bound ^{VII}	Outward facing	CM3	7 / 4 / 4	3.62 / 3.66 / 3.64
		CM8	109 / 107 / 114	3.67 / 3.76 / 3.62
		CM10	38 / 49 / 49	4.89 / 4.65 / 5.06
		CM11	16 / 18 / 14	2.93 / 3.21 / 3.09

Table A17 Results for MMM analysis of Glt_{ph} variants spin labelled with MTSL. All five variants were analysed giving predictions of the number of rotamers on each protomer (A/B/C) and the distance distributions between pairs of residues (AB, AC, BC). A range of binding states and conformations were studied to see how the selected position moves during the transport cycle where substrate bound refers to sodium and L-aspartate, and inhibitor is with sodium and DL-TBOA. ^IPDB 4OYE, ^{II}PDB 4P19, ^{III}PDB 4OYF, ^{IV}PDB 3KBC, ^VPDB 6X15, ^{VI}PDB 6X16, ^{VII}PDB 2NWW.

State	Conformation(s)	Variant	Rotamers of NTA	Distances (nm)
Apo ^I	Outward facing occluded	CM3	x	
		CM7 & CM8	11 / 12 / 11	3.25 / 3.26 / 3.26
		CM10	x	
		CM11	x	
Apo ^{II}	Inward facing occluded	CM3	x	
		CM7 & CM8	6 / 8 / 9	3.38 / 3.37 / 3.40
		CM10	41 / 42 / 43	2.92 / 2.91 / 2.89
		CM11	5 / 3 / 3	2.70 / 2.85 / 2.90
Na ⁺ only ^{III}	Outward occluded	CM3	x	
		CM7 & CM8	16 / 16 / 14	3.23 / 3.22 / 3.22
		CM10	72 / 68 / 72	2.95 / 2.94 / 2.95
		CM11	17 / 16 / 17	4.26 / 4.24 / 4.27
Substrate bound ^{IV}	Inward facing occluded	CM3	x	
		CM7 & CM8	18 / 17 / 18	3.46 / 3.46 / 3.45
		CM10	69 / 69 / 70	2.80 / 2.80 / 2.81
		CM11	13 / 13 / 13	2.63 / 2.63 / 2.62
Substrate bound ^V	Inward facing	CM3	1 / 1 / 1	4.40 / 4.35 / 4.40
		CM7 & CM8	5 / 5 / 5	3.42 / 3.42 / 3.42
		CM10	91 / 91 / 91	2.96 / 2.96 / 2.97
		CM11	25 / 25 / 25	2.66 / 2.65 / 2.65
Inhibitor bound ^{VI}	Inward facing	CM3	x	
		CM7 & CM8	6 / 6 / 6	3.36 / 3.37 / 3.39
		CM10	67 / 78 / 67	2.99 / 2.94 / 2.99
		CM11	29 / 29 / 29	2.67 / 2.67 / 2.69
Inhibitor bound ^{VII}	Outward facing	CM3	x	
		CM7 & CM8	7 / 8 / 9	3.34 / 3.31 / 3.30
		CM10	48 / 43 / 51	2.85 / 2.89 / 2.84
		CM11	7 / 1 / 9	4.36 / 4.51 / 4.47

Table A18 Results for MMM analysis of Glt_{ph} variants spin labelled with Cu²⁺-NTA. All five variants were analysed giving predictions of the number of rotamers on each protomer (A/B/C) and the distance distributions between pairs of residues (AB, AC, BC). A range of binding states and conformations were studied to see how the selected position moves during the transport cycle where substrate bound refers to sodium and L-aspartate, and inhibitor is with sodium and DL-TBOA. ^IPDB 4OYE, ^{II}PDB 4P19, ^{III}PDB 4OYF, ^{IV}PDB 3KBC, ^VPDB 6X15, ^{VI}PDB 6X16, ^{VII}PDB 2NWW

State	Conformation(s)	Variant	Distances (nm)
Apo ^I	Outward facing	CM7	3.08 / 2.37 / 2.36 / 3.08 / 3.08 / 2.35
	occluded	CM8	4.97 / 3.98 / 3.97 / 5.00 / 4.99 / 3.99
Apo ^{II}	Inward facing	CM7	2.72 / 2.48 / 2.42 / 2.82 / 2.67 / 2.41
		CM8	2.96 / 3.22 / 3.24 / 2.97 / 3.00 / 3.28
	occluded	CM10	3.66 / 4.58 / 4.69 / 3.74 / 3.60 / 3.51
		CM11	2.59 / 3.13 / 3.06 / 2.53 / 2.53 / 3.26
Na ⁺ only ^{III}	Outward occluded	CM7	3.20 / 2.40 / 2.38 / 3.17 / 3.19 / 2.39
		CM8	4.96 / 3.85 / 3.86 / 4.97 / 4.96 / 3.85
		CM10	3.32 / 4.03 / 3.96 / 3.26 / 3.35 / 4.07
		CM11	2.72 / 4.42 / 4.45 / 2.69 / 2.71 / 4.40
Substrate bound ^{IV}	Inward facing	CM7	3.01 / 2.68 / 2.68 / 3.01 / 3.02 / 2.66
		CM8	2.85 / 3.00 / 2.97 / 2.83 / 2.86 / 2.98
	occluded	CM10	2.72 / 3.77 / 3.75 / 2.71 / 2.71 / 3.78
		CM11	2.37 / 3.20 / 3.17 / 2.39 / 2.37 / 3.16
Substrate bound ^V	Inward facing	CM3	5.00 / 4.87 / 4.87 / 5.01 / 5.00 / 4.87
		CM7	3.20 / 2.62 / 2.62 / 3.19 / 3.20 / 2.62
		CM8	2.75 / 3.00 / 3.01 / 2.75 / 2.74 / 3.01
		CM10	3.29 / 4.00 / 4.00 / 3.29 / 3.29 / 3.99
		CM11	2.43 / 3.27 / 3.29 / 2.42 / 2.41 / 3.28
Inhibitor bound ^{VI}	Inward facing	CM7	2.93 / 2.19 / 2.04 / 2.91 / 2.88 / 2.08
		CM8	2.70 / 2.76 / 2.74 / 2.72 / 2.69 / 2.77
		CM10	3.68 / 4.44 / 4.46 / 3.68 / 3.68 / 4.46
		CM11	2.49 / 3.19 / 3.16 / 2.47 / 2.46 / 3.20
Inhibitor bound ^{VII}	Outward facing	CM7	3.22 / 2.43 / 2.32 / 3.08 / 3.35 / 2.49
		CM8	4.89 / 3.49 / 3.45 / 4.78 / 4.88 / 3.48
		CM10	3.51 / 4.03 / 4.30 / 3.60 / 3.61 / 4.35
		CM11	2.34 / 4.45 / 4.57 / 2.46 / 2.59 / 4.59

Table A19 Results for MMM analysis of GlT_{ph} variants spin labelled with Cu²⁺-NTA and MTSL. All five variants were analysed giving the inter-protomer distance distributions between pairs of Cu²⁺-NTA and MTSL residues (AB, AC, BC). A range of binding states and conformations were studied to see how the selected positions move during the transport cycle relative to each other, where substrate bound refers to sodium and L-aspartate, and inhibitor is with sodium and DL-TBOA. ^IPDB 4OYE, ^{II}PDB 4P19, ^{III}PDB 4OYF, ^{IV}PDB 3KBC, ^VPDB 6X15, ^{VI}PDB 6X16, ^{VII}PDB 2NWW

State	Conformation(s)	Variant	Distances (nm)
Apo ^I	Outward facing	CM7	1.41 / 1.41 / 1.41
	occluded	CM8	<u>3.40 / 3.40 / 3.40</u>
Apo ^{II}	Inward facing	CM7	1.32 / 1.30 / 1.34
		CM8	1.23 / 1.28 / 1.22
	occluded	CM10	<u>3.46 / 3.51 / 3.44</u>
		CM11	1.12 / 1.24 / 1.12
Na ⁺ only ^{III}	Outward occluded	CM7	1.34 / 1.34 / 1.33
		CM8	<u>3.32 / 3.31 / 3.31</u>
		CM10	1.33 / 1.34 / 1.36
		CM11	<u>2.51 / 2.54 / 2.51</u>
Substrate bound ^{IV}	Inward facing occluded	CM7	1.44 / 1.45 / 1.45
		CM8	1.28 / 1.27 / 1.28
		CM10	1.72 / 1.73 / 1.73
		CM11	1.25 / 1.27 / 1.25
Substrate bound ^V	Inward facing	CM3	<u>2.15 / 2.15 / 2.15</u>
		CM7	1.50 / 1.51 / 1.50
		CM8	1.24 / 1.24 / 1.24
		CM10	<u>2.27 / 2.27 / 2.27</u>
		CM11	1.30 / 1.31 / 1.31
Inhibitor bound ^{VI}	Inward facing	CM7	1.61 / 1.60 / 1.60
		CM8	1.28 / 1.28 / 1.28
		CM10	<u>3.36 / 3.36 / 3.36</u>
		CM11	1.21 / 1.21 / 1.21
Inhibitor bound ^{VII}	Outward facing	CM7	1.40 / 1.44 / 1.44
		CM8	<u>3.28 / 3.28 / 3.35</u>
		CM10	1.24 / 1.57 / 1.65
		CM11	<u>3.01 / 2.80 / 2.78</u>

Table A20 Results for MMM analysis of GlT_{ph} variants spin labelled with Cu²⁺-NTA and MTSL. All five variants were analysed giving the intra-protomer distance distributions between pairs of Cu²⁺-NTA and MTSL residues on each protomer. A range of binding states and conformations were studied to see how the selected positions move during the transport cycle relative to each other, where substrate bound refers to sodium and L-aspartate, and inhibitor is with sodium and DL-TBOA. Underlined distances highlight those within the range for distance determination using DEER experiments. ^IPDB 4OYE, ^{II}PDB 4P19, ^{III}PDB 4OYF, ^{IV}PDB 3KBC, ^VPDB 6X15, ^{VI}PDB 6X16, ^{VII}PDB 2NWW.

A17 *In silico* attachment of Cu²⁺-NTA to an engineered dHis motif on Glt_{Ph} using Chimera and MMM

The start of this method closely follows that of MTSL-labelling using MMM as detailed in the materials and methods chapter.

In MMM, a PDB file is loaded: *File – New from PDB/web*

The two histidine residues in the dHis motif must be selected, spin-labelled and rotamers attached.

- *Select (A,B,C)51,55 ...* which selects residues 51 and 55 on the three protomers A-C
- *EPR - site scan selected residues – ok – select dHis-Cu as the label – ok*
- *EPR – attach precomputed rotamers – select dHis-Cu as the label – ok*

Save as a PDB file which can then be opened in Chimera.

In Chimera, the dHis motif needs to be shown, and the copper atom built in

- *Select – residue – HCU – Actions – atoms/bonds – show*
- In the command line: *sel:HCU:A ...* which selects the dHis motif on protomer A
- Centre the view (in the command line): *centre sel*
- Then add in the copper atom which goes between the centred selection i.e. the dHis motif selected on A
Tools – structure editing – start structure – modify structure – change element to Cu, bonds = 0.
Actions – inspect – atom style = ball, radius = 1.28
- Repeat for other protomers as needed.

A18 Continuous wave EPR spectroscopy of Glt_{ph}

Functional studies were conducted using room temperature (RT) cw-EPR spectroscopy for each of the Glt_{ph} variants in the four binding conditions and are presented below. Changes in line shape observed between the four binding conditions indicated the protein had undergone conformational changes as observed for all Glt_{ph} variants to different extents. Glt_{ph} CM11 showed the most subtle line shape changes indicating there were either no or very subtle changes in the spin label environment during the transport cycle. Glt_{ph} CM8 displayed more significant line shape changes which may correspond to larger changes in the spin label environment.

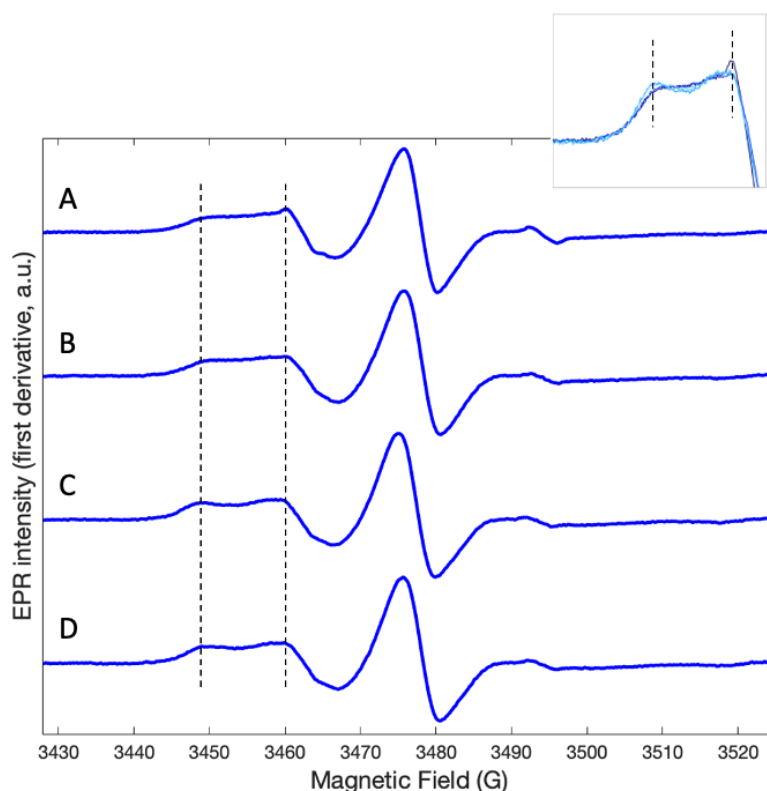


Figure A30 X-band RT cw-EPR spectra of Glt_{ph} CM3 in detergent micelles utilising substrate and co-transported Na⁺ to trap Glt_{ph} in proposed intermediates of the transport cycle. A) apo, B) Na⁺ (50 mM), C) substrate bound (50 mM Na⁺ and 1 mM L-aspartate) and D) inhibitor bound (50 mM Na⁺ and 1 mM DL-TBOA). Black dashed lines indicate regions of the spectra where subtle changes are observed (see insert). Zoomed in area of the biggest shape changes in the binding conditions shown darkest to lightest for A) to D). Experimental parameters: microwave frequency = 9.758 GHz, field modulation frequency = 100 kHz, modulation amplitude = 2G, microwave power = 0.1997 mW, number averages = 100, time constant = 20.48 ms, conversion time = 20.48 ms.

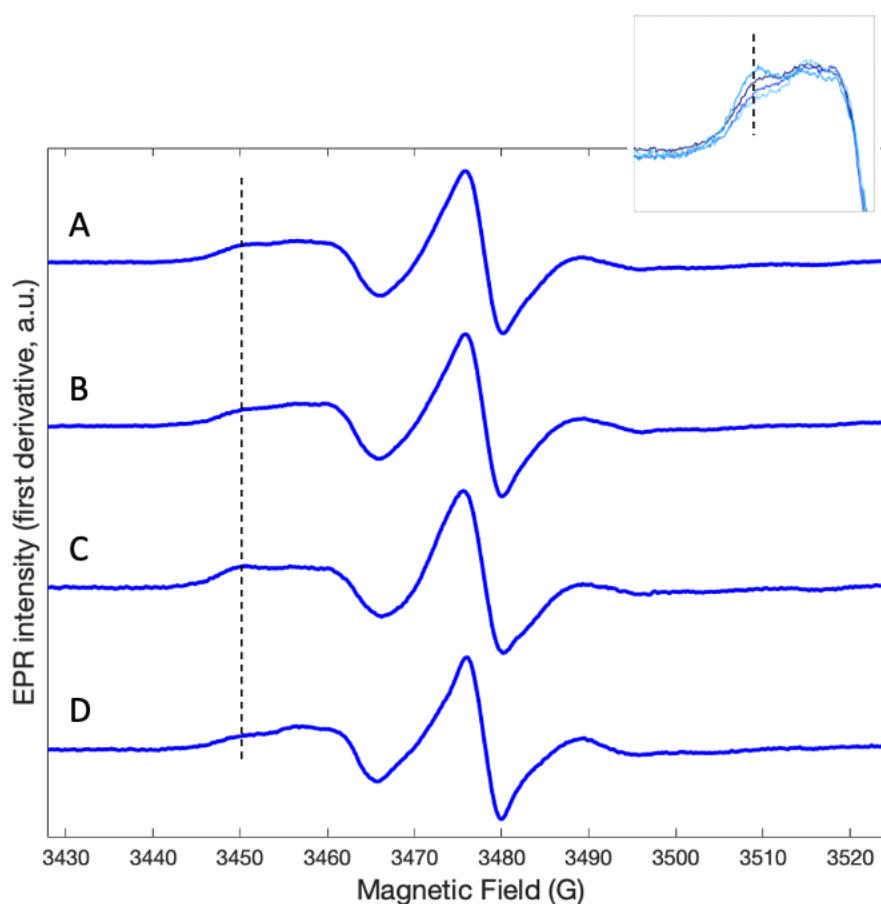


Figure A31 X-band RT cw-EPR spectra of Glt_{ph}CM8 in detergent micelles utilising substrate and co-transported Na⁺ to trap Glt_{ph} in proposed intermediates of the transport cycle. A) apo, B) Na⁺ (50 mM), C) substrate bound (50 mM Na⁺ and 1 mM L-aspartate) and D) inhibitor bound (50 mM Na⁺ and 1 mM DL-TBOA). Black dashed lines indicate regions of the spectra where subtle changes are observed (see insert). Zoomed in area of the biggest shape changes in the binding conditions shown darkest to lightest for A) to D). Experimental parameters: microwave frequency = 9.758 GHz, field modulation frequency = 100 kHz, modulation amplitude = 2G, microwave power = 0.1997 mW, number averages = 100, time constant = 20.48 ms, conversion time = 20.48 ms.

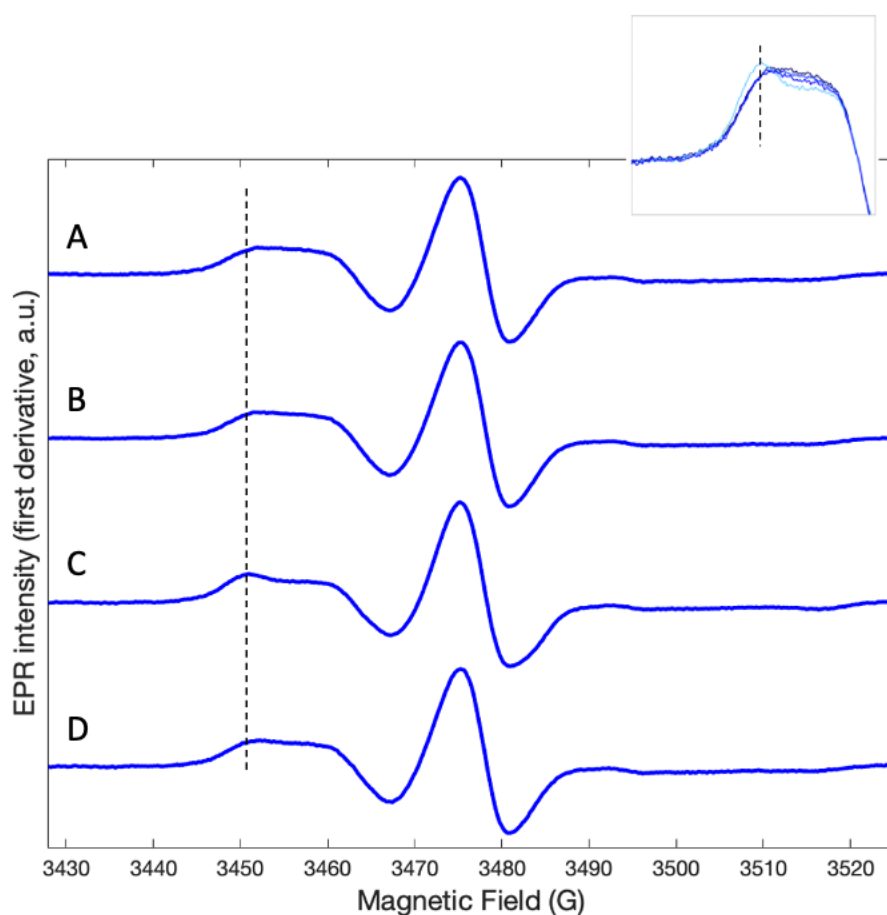


Figure A32 X-band RT cw-EPR spectra of Glt_{ph} CM10 in detergent micelles utilising substrate and co-transported Na⁺ to trap Glt_{ph} in proposed intermediates of the transport cycle. A) apo, B) Na⁺ (50 mM), C) substrate bound (50 mM Na⁺ and 1 mM L-aspartate) and D) inhibitor bound (50 mM Na⁺ and 1 mM DL-TBOA). Black dashed lines indicate regions of the spectra where subtle changes are observed (see insert). Zoomed in area of the biggest shape changes in the binding conditions shown darkest to lightest for A) to D). Experimental parameters: microwave frequency = 9.758 GHz, field modulation frequency = 100 kHz, modulation amplitude = 2G, microwave power = 0.1997 mW, number averages = 100, time constant = 20.48 ms, conversion time = 20.48 ms.

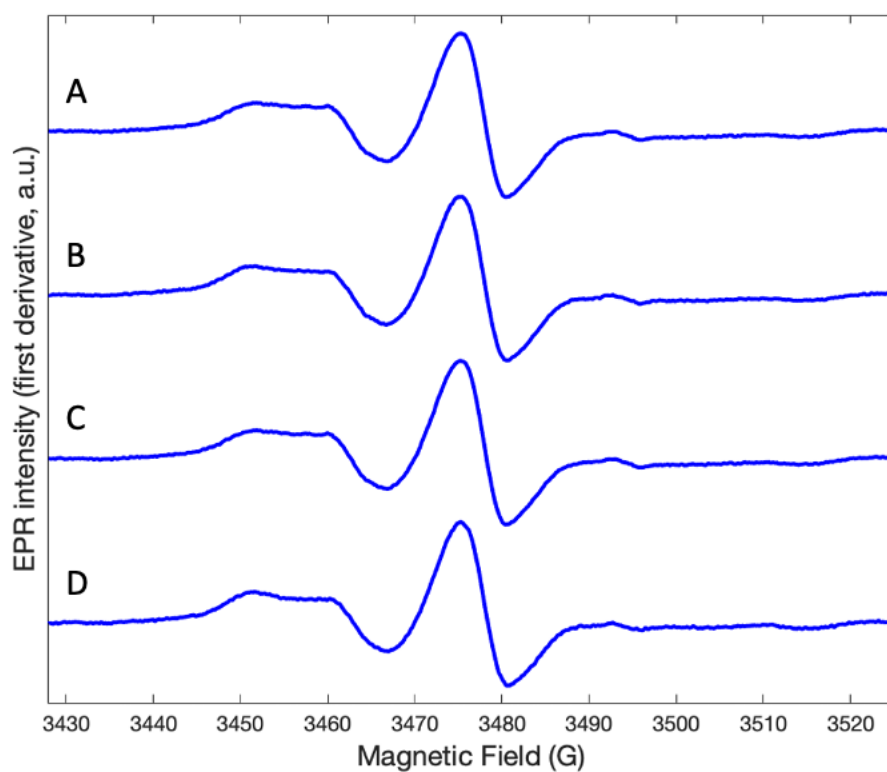


Figure A33 X-band RT cw-EPR spectra of Glt_{ph} CM11 in detergent micelles utilising substrate and co-transported Na⁺ to trap Glt_{ph} in proposed intermediates of the transport cycle. A) apo, B) Na⁺ (50 mM), C) substrate bound (50 mM Na⁺ and 1 mM L-aspartate) and D) inhibitor bound (50 mM Na⁺ and 1 mM DL-TBOA). Experimental parameters: microwave frequency = 9.758 GHz, field modulation frequency = 100 kHz, modulation amplitude = 2G, microwave power = 0.1997 mW, number averages = 100, time constant = 20.48 ms, conversion time = 20.48 ms.

A19 Glt_{ph} 4-pulse DEER results for CM3, CM8, CM10 and CM11

The MMM results for Glt_{ph} based on published structures showed no specific correlation between particular conformations and distances (Table A16). The inward occluded state of apo-protein compared to substrate bound predicted different distance distributions which for some variants varied quite significantly. This made correlating the distance distributions calculated using DeerNet with specific conformations difficult. So each binding state was compared to the predicted MMM results in the same binding conditions. All samples showed a long distance at the cut-off point – i.e. for 2.5 μ s time decay this was at 5 nm, and for 1.5 μ s time decay this was at 4 nm. This peak didn't change in intensity or distance between each set of samples with the same time decay. These peaks may be an artefact introduced from the simulation of the background corrected time decay as a result of high noise levels at the end. These peaks have been discounted from the analysis of the distance distribution results, and in all cases did not correspond to any distances predicted by MMM.

A19.1 Glt_{ph} CM3 DEER results

The 4-pulse DEER results for Glt_{ph} CM3 showed subtle changes in distance distributions between the four binding conditions, where multiple conformations were present for each. DeerNet calculated distances of 4 nm for apo Glt_{ph} CM3, with the 5 nm distances discounted as mentioned above. The broad distance distribution at 4 nm was close to the predicted distance of 4.5 nm for an inward occluded conformation in an apo state. The outward occluded conformation was predicted to have a much shorter distance of 3.2 nm which was not seen. The population of the broad 4.0 nm distance increased on binding Na⁺ and became a more distinct peak when substrate bound. MMM predictions for substrate bound Glt_{ph} CM3 included 4.1 nm (inward occluded) and 4.5 nm (inward facing) both of which may account for the broad distance distribution observed – with a larger population of the inward occluded conformation. The substrate bound outward-occluded state was predicted to have a distance of 3.9 nm which could also be present as an underlying part of the

broad distance distribution. Inhibitor bound Glt_{ph} CM3 displayed a main distance distribution at 3.9 nm (ignoring the 5 nm peak) which corresponds to the outward facing MMM predictions.

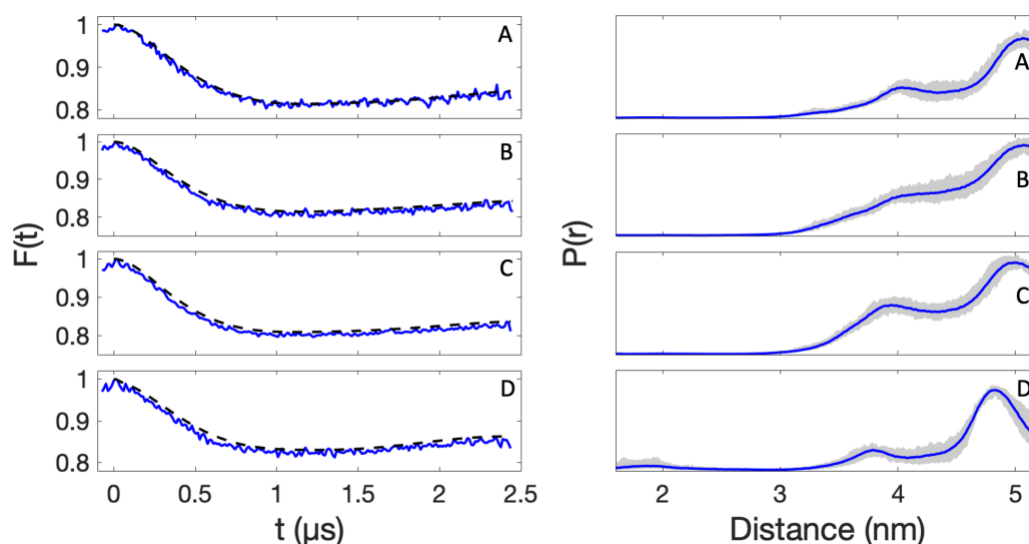


Figure A34 4-pulse DEER experiments of MTSL-labelled Glt_{ph} CM3 in detergent micelles utilising substrate and co-transported Na⁺ to trap Glt_{ph} in proposed intermediates of the transport cycle. A) apo, B) 50 mM Na⁺, C) substrate bound (50 mM Na⁺ & 1 mM L-aspartate), and D) inhibitor bound (50 mM Na⁺ & 1 mM DL-TBOA). The normalised background corrected time decays are shown left with their respective fittings shown as black dashed lines. The right hand spectra show the calculated distance distributions using DeerNet and the 95% confidence bounds represented by the light grey areas.

A19.2 Glt_{ph} CM8 DEER results

Glt_{ph} CM8 had a very shallow modulation depth of around 10% indicating each sample had a weak signal. The background corrected time decays were relatively featureless with no clear oscillations present which would correspond to short distance distributions. The predicted distances from MMM ranged between 1.9–3.9 nm, where the shorter distance should be visible as an oscillation in the raw data. For apo Glt_{ph} CM8 there was a large distance distribution between 2.5–4 nm which covers the distances predicted for both an outward occluded (3.6 nm) and inward occluded (2.6 nm) conformation. These results suggest the protein was sampling multiple conformations. Addition of Na⁺ resulted in more distinct peaks at 2.6 nm and

3.7 nm which may correspond to the same conformations as apo Glt_{ph} CM8. A new distance at 2.3 nm appeared when substrate bound. Interestingly the MMM predictions for three conformations in this binding state were all around 2.3 nm: inward-facing (2.2 nm), inward occluded (2.3 nm) and outward occluded (2.2 nm). This highlights how same conformation in different binding states has distinctive distances, and different conformations were predicted to have the same distances. Based on the apo and Na⁺ results, the outward occluded conformation and inward occluded conformations may represent the peaks at 3.6 nm and 2.6 nm respectively, with the new shorter distance at 2.3 nm corresponding to an inward facing state. Inhibitor bound Glt_{ph} CM8 showed a single distance around 3.8–4 nm which corresponds to the MMM prediction for outward facing state at 3.8 nm.

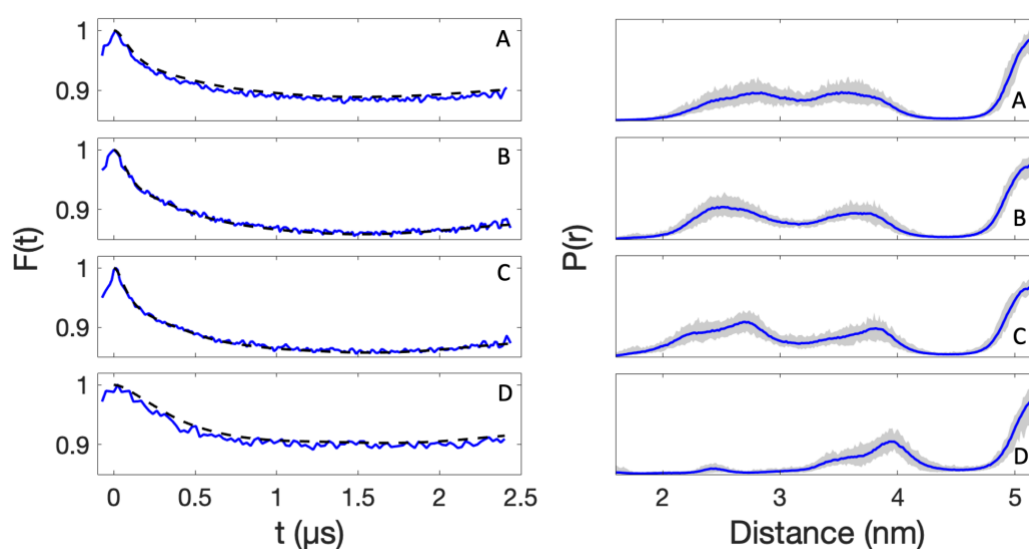


Figure A35 4-pulse DEER experiments of MTSL-labelled Glt_{ph} CM8 in detergent micelles utilising substrate and co-transported Na⁺ to trap Glt_{ph} in proposed intermediates of the transport cycle. A) apo, B) 50 mM Na⁺, C) substrate bound (50 mM Na⁺ & 1 mM L-aspartate), and D) inhibitor bound (50 mM Na⁺ & 1 mM DL-TBOA). The normalised background corrected time decays are shown left with their respective fittings shown as black dashed lines. The right hand spectra show the calculated distance distributions using DeerNet and the 95% confidence bounds represented by the light grey areas.

A19.3 Glt_{ph} CM10 DEER results

The extracted distance distributions from the background corrected time decay spectra for Glt_{ph} CM10 were low in relative population. The MMM predicted distances ranged between 2.6–4.5 nm which should easily be visible using DEER experiments. For apo Glt_{ph} CM10, a distance distribution of 3.8 nm was calculated. However, the two structures available for this state predicted distances of 4.2 nm (for outward occluded) and 3.0 nm (for inward occluded). The most similar distance distribution predicted was for inward occluded substrate bound Glt_{ph} at 3.5 nm. The distance distribution shifted slightly shorter upon addition of Na⁺ and broadened between 3.3 - 3.8 nm for substrate-bound Glt_{ph} CM10. Inward facing substrate bound Glt_{ph} CM10 was predicted to have a distance of 3.2 nm which could correspond to the lower end of this range. The time decay for inhibitor bound Glt_{ph} CM10 was poor, resulting in a featureless distance distribution which could not be interpreted. Overall, these results showed subtle changes in the distance distributions of Glt_{ph} CM10, but the low modulation depth significantly limited the results.

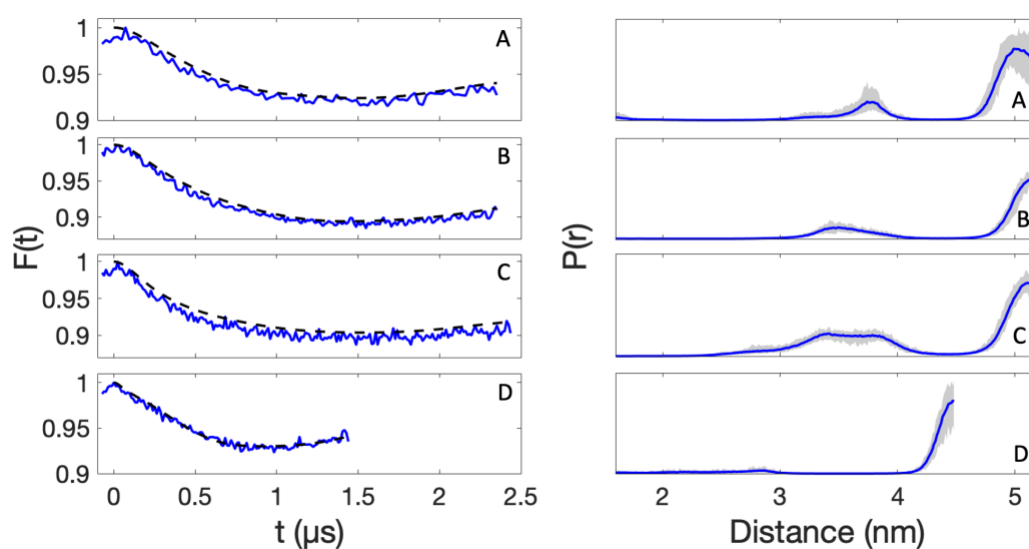


Figure A36 4-pulse DEER experiments of MTS-labeled GltPh CM10 in detergent micelles utilising substrate and co-transported Na⁺ to trap Glt_{ph} in proposed intermediates of the transport cycle. A) apo, B) 50 mM Na⁺, C) substrate bound (50 mM Na⁺ & 1 mM L-aspartate), and D) inhibitor bound (50 mM Na⁺ & 1 mM DL-TBOA). The normalised background corrected time decays are shown left with their respective fittings shown as black dashed lines. The right hand spectra show the calculated distance distributions using DeerNet and the 95% confidence bounds represented by the light grey areas.

A19.4 Glt_{ph} CM11 DEER results

The predicted distance distributions for Glt_{ph} CM11 were very similar for different conformations and binding states (ranging between 2.8–3.2 nm) which made deconvolution of the results challenging. This could also account for the lack of line shape changes seen in the RT cw-EPR spectra (Figure A30). For substrate bound Glt_{ph} CM11, the inward facing, inward occluded and outward occluded conformations all predicted the same distances of ~3.0 nm making assignment of a particular conformational state impossible based on distances alone. The shallow modulation depths of all four binding conditions also indicated the sample had a poor signal and hence resulted in time decays with high noise levels. This meant it was harder to analyse the data and extract distance distributions using DeerNet and could result in the introduction of artificial distances. A distance of 2.8 nm appeared in all four binding states and is proposed to correspond to an occluded conformation based on MMM predictions. There were no predicted distances <2.8 nm which could account for the short distances seen in substrate and inhibitor bound samples. This distance may be a result of simulation noisy data using DeerNet causing it to be artificially introduced. Overall, this variant showed very little change in distance distribution.

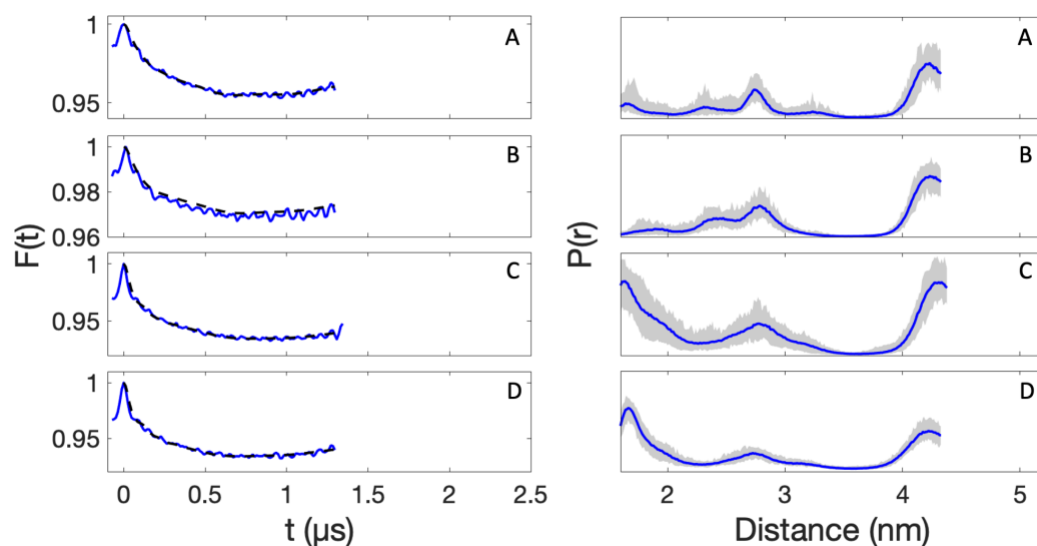


Figure A37 4-pulse DEER experiments of MTSL-labelled GltPh CM11 in detergent micelles utilising substrate and co-transported Na^+ to trap Glt_{ph} in proposed intermediates of the transport cycle. A) apo, B) 50 mM Na^+ , C) substrate bound (50 mM Na^+ & 1 mM L-aspartate), and D) inhibitor bound (50 mM Na^+ & 1 mM DL-TBOA). The normalised background corrected time decays are shown left with their respective fittings shown as black dashed lines. The right hand spectra show the calculated distance distributions using DeerNet and the 95% confidence bounds represented by the light grey areas.

A20 Continuous wave EPR spectroscopy of Glt_{ph}: His-tag cleavage control check

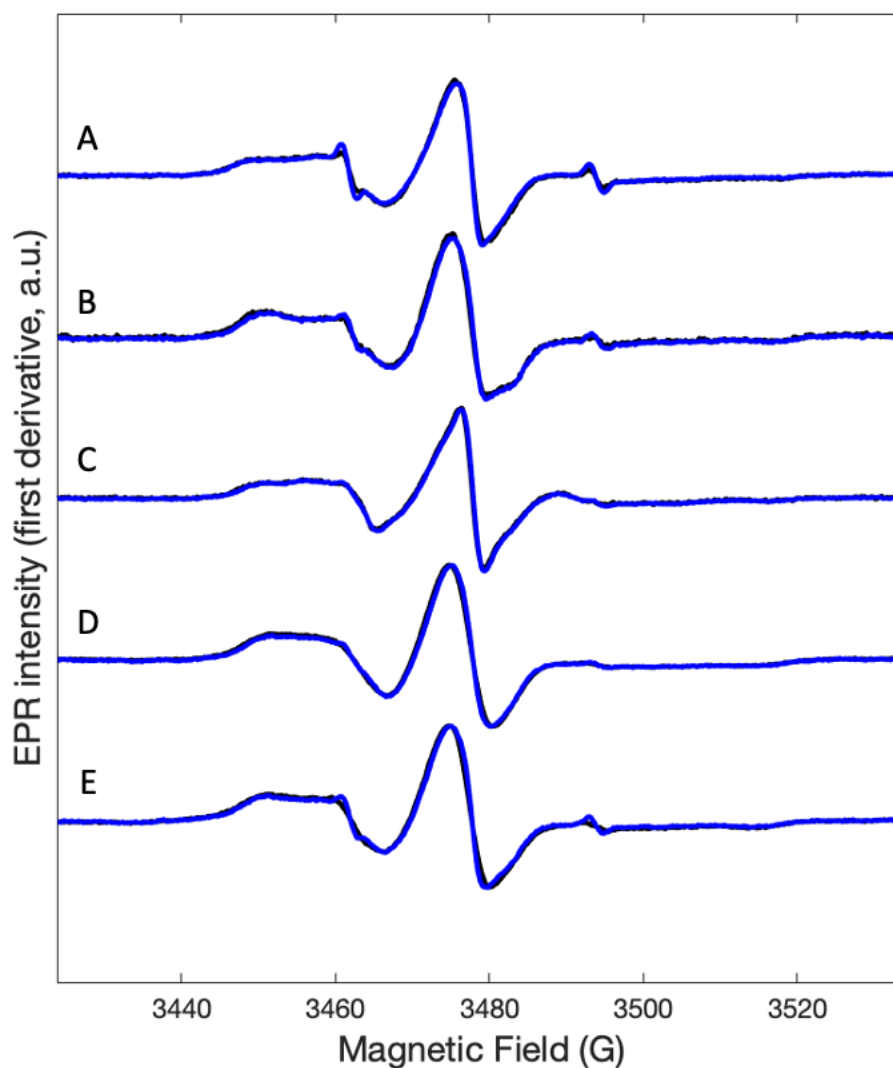


Figure A38 X-band RT cw-EPR spectra of all Glt_{ph} variants in detergent micelles in an apo state (blue) and following poly-histidine tag cleavage (black). A) CM3, B) CM7, C) CM8, D) CM10 and E) CM11. There was no change in line shape between cleaved and un-cleaved protein. Experimental parameters: microwave frequency = 9.758 GHz, field modulation frequency = 100 kHz, modulation amplitude = 2G, microwave power = 0.1997 mW, number averages = 100, time constant = 20.48 ms, conversion time = 20.48 ms.

A21 A typical LT X-band spectrum of Cu

The hyperfine coupling along g -parallel ($g_{\parallel}$) for Cu^{2+} is always much greater than g -perpendicular ($g_{\perp}$), resulting in large splitting of $g_{\parallel}$ line and often unobservable splitting of $g_{\perp}$.

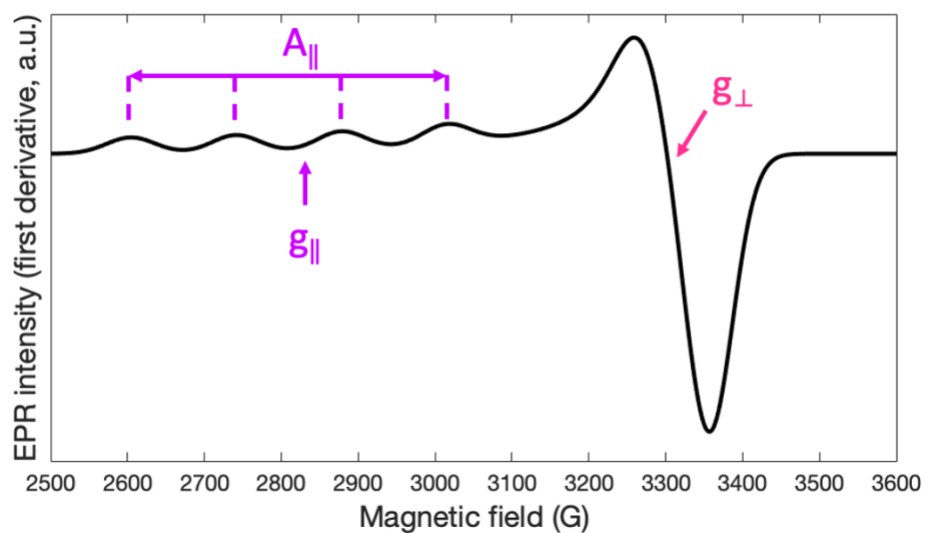


Figure A39 EPR spectrum of Cu^{2+} at LT X-band

A22 The T_2 spectrum of Glt_{ph} CM7 spin-labelled with MTSL, Cu²⁺-NTA and orthogonally labelled

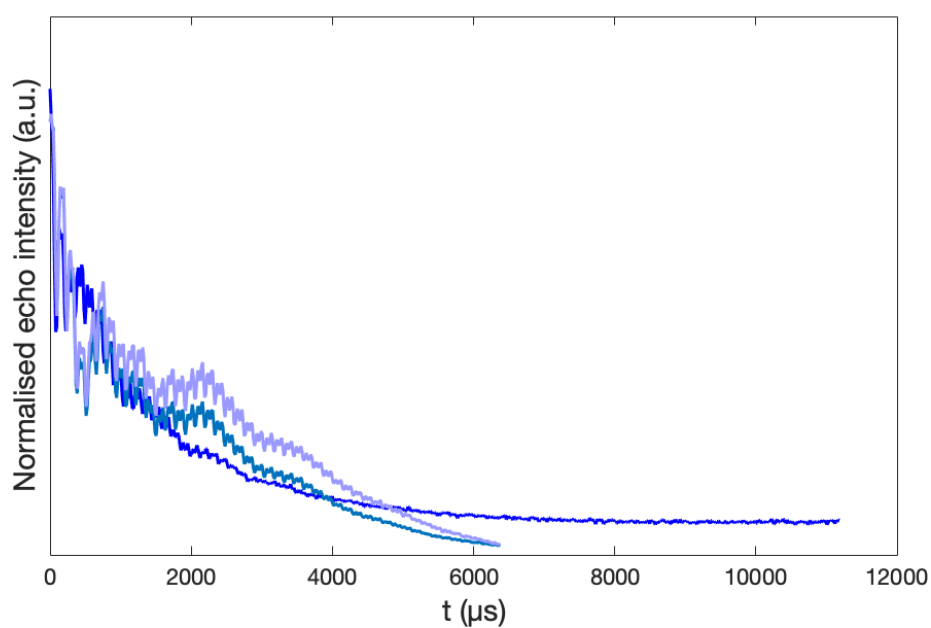


Figure A40 T_2 experiment of Glt_{ph} CM7 spin labelled with MTSL (bright blue), Cu²⁺-NTA (blue) and orthogonally labelled with both (lilac) – all in the presence of 30% v/v d₈-glycerol.